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(54) Title: METHODS FOR USING COMPOSITIONS COMPRISING HEAT SHOCK PROTEINS OR ALPHA-2-MACROGLOBULIN IN THE TREATMENT OF CANCER AND INFECTIOUS DISEASE

(57) Abstract: The present invention relates to methods and compositions for the prevention and treatment of infectious diseases, and cancers. The methods of the invention comprises administering (a) a composition comprising a population of complexes of antigenic proteins or antigenic peptides derived from antigenic cells or viral particles and one or more different heat shock proteins; and (b) a non-heat shock protein and non-alpha-2-macroglobulin-based treatment modality. The population or the protein preparation used to produce the antigenic peptides comprises at least 50% of the different proteins or at least 50 different proteins of the antigenic cells or viral particles. Methods for making antigenic peptides comprise digesting a protein preparation of antigenic cells, a cellular fraction thereof, or of viral particles with one or more proteases, or exposing the protein preparation to ATP, guanidium hydrochloride, and/or acidic conditions.

# METHODS FOR USING COMPOSITIONS COMPRISING HEAT SHOCK PROTEINS OR ALPHA-2-MACROGLOBULIN IN THE TREATMENT OF CANCER AND INFECTIOUS DISEASE

This invention was made with government support under grant number CA/A184479 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

## 1. INTRODUCTION

The present invention relates to methods and compositions for the prevention and treatment of infectious diseases, and primary and metastatic neoplastic diseases. In the practice of the prevention and treatment of infectious diseases and cancer, compositions comprising cytosolic and membrane-derived proteins from antigenic cells and/or the digestion products thereof, are complexed to heat shock proteins and/or alpha-2-macroglobulin to augment the immune response to tumors and infectious agents. The uses of such compositions in combination with other treatment modalities are also encompassed.

## 2. BACKGROUND OF THE INVENTION

### 2.1. HEAT SHOCK PROTEINS

Heat shock proteins (HSPs), also referred to as stress proteins, were first identified as proteins synthesized by cells in response to heat shock. HSPs have been classified into five families, based on molecular weight, HSP100, HSP90, HSP70, HSP60, and smHSP. Many members of these families were found subsequently to be induced in response to other stressful stimuli including nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens (see Welch, May 1993, Scientific American 56-64; Young, 1990, Annu. Rev. Immunol. 8:401-420; Craig, 1993, Science 260:1902-1903; Gething *et al.*, 1992, Nature 355:33-45; and Lindquist *et al.*, 1988, Annu. Rev. Genetics 22:631-677).

Studies on the cellular response to heat shock and other physiological stresses revealed that the HSPs are involved not only in cellular protection against these adverse conditions, but also in essential biochemical and immunological processes in unstressed cells.

HSPs accomplish different kinds of chaperoning functions. For example, members of the HSP70 family, located in the cell cytoplasm, nucleus, mitochondria, or endoplasmic reticulum (Lindquist *et al.*, 1988, Ann. Rev. Genetics 22:631-677), are involved in the presentation of antigens to the cells of the immune system, and are also involved in the transfer, folding and assembly of proteins in normal cells. HSPs are capable of binding proteins or peptides, and releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or acidic conditions (Udono and Srivastava, 1993, J. Exp. Med. 178:1391-1396).

Srivastava *et al.* demonstrated immune response to methylcholanthrene-induced sarcomas of inbred mice (1988, Immunol. Today 9:78-83). In these studies, it was found that the molecules responsible for the individually distinct immunogenicity of these tumors were glycoproteins of 96kDa (gp96) and intracellular proteins of 84 to 86kDa (Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3407-3411; Ullrich *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3121-3125). Immunization of mice with gp96 or p84/86 isolated from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically distinct tumors. Isolation and characterization of genes encoding gp96 and p84/86 revealed significant homology between them, and showed that gp96 and p84/86 were, respectively, the endoplasmic reticular and cytosolic counterparts of the same heat shock proteins (Srivastava *et al.*, 1988, Immunogenetics 28:205-207; Srivastava *et al.*, 1991, Curr. Top. Microbiol. Immunol. 167:109-123). Further, HSP70 was shown to elicit immunity to the tumor from which it was isolated but not to antigenically distinct tumors. However, HSP70 depleted of peptides was found to lose its immunogenic activity (Udono and Srivastava, 1993, J. Exp. Med. 178:1391-1396). These observations suggested that the heat shock proteins are not immunogenic per se, but form noncovalent complexes with antigenic peptides, and the complexes can elicit specific immunity to the antigenic peptides (Srivastava, 1993, Adv. Cancer Res. 62:153-177; Udono *et al.*, 1994, J. Immunol., 152:5398-5403; Suto *et al.*, 1995, Science 269:1585-1588).

Noncovalent complexes of HSPs and peptide, purified from cancer cells, can be used for the treatment and prevention of cancer and have been described in PCT publications WO 96/10411, dated April 11, 1996, and WO 97/10001, dated March 20, 1997 (U.S. Patent No. 5,750,119 issued May 12, 1998, and U.S. Patent No. 5,837,251 issued November 17, 1998,

respectively, each of which is incorporated by reference herein in its entirety). The isolation and purification of HSP-peptide complexes has been described, for example, from pathogen-infected cells, and used for the treatment and prevention of infection caused by the pathogen, such as viruses, and other intracellular pathogens, including bacteria, protozoa, fungi and parasites (see, for example, PCT Publication WO 95/24923, dated September 21, 1995). Immunogenic stress protein-antigen complexes can also be prepared by in vitro complexing of stress protein and antigenic peptides, and the uses of such complexes for the treatment and prevention of cancer and infectious diseases has been described in PCT publication WO 97/10000, dated March 20, 1997 (U.S. Patent No. 6,030,618 issued February 29, 2000). The use of stress protein-antigen complexes for sensitizing antigen presenting cells in vitro for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997 (see also U.S. Patent No. 5,985,270 issued November 16, 1999).

## 2.2. ALPHA-2-MACROGLOBULIN

The α-macroglobulins are members of a protein superfamily of structurally related proteins which also comprises complement components C3, C4 and C5. The human plasma protein alpha-2-macroglobulin (α2M) is a 720 kDa homotetrameric protein primarily known as proteinase inhibitor and plasma and inflammatory fluid proteinase scavenger molecule (for review see Chu and Pizzo, 1994, Lab. Invest. 71:792). α2M is synthesized as a precursor having 1474 amino acid residues. The first 23 amino acids function as a signal sequence that is cleaved to yield a mature protein with 1451 amino acid residues (Kan *et al.*, 1985, Proc. Natl. Acad. Sci. U.S.A. 82:2282-2286).

α2M promiscuously binds to proteins and peptides with nucleophilic amino acid side chains in a covalent manner (Chu *et al.*, 1994, Ann. N.Y. Acad. Sci. 737:291-307) and targets them to cells which express a α2M receptor (α2MR) (Chu and Pizzo, 1993, J. Immunol. 150:48). Binding of α2M to the α2M receptor is mediated by the carboxy-terminal portion of α2M (Holtet *et al.*, 1994, FEBS Lett. 344:242-246) and key residues have been identified (Nielsen et al., 1996, J. Biol. Chem. 271:12909-12912).

Generally known for inhibiting protease activity, α2M binds to a variety of proteases through multiple binding sites (*see, e.g.*, Hall *et al.*, 1981, Biochem. Biophys. Res. Commun. 100(1):8-16). Protease interaction with α2M results in a complex structural rearrangement called

transformation, which is the result of a cleavage within the "bait" region of α2M after the proteinase becomes "trapped" by thioesters. The conformational change exposes residues required for receptor binding, allowing the α2M-proteinase complex to bind to the α2MR. Methylamine can induce similar conformational changes and cleavage as that induced by proteinases. The uncleaved form of α2M, which is not recognized by the receptor, is often referred to as the "slow" form (s-α2M). The cleaved form is referred to as the "fast" form (f-α2M) (reviewed by Chu *et al.*, 1994, Ann. N.Y. Acad. Sci. 737:291-307). Recently, it has also been shown that the α2MR can bind to HSPs, such as gp96, hsp90, hsp70, and calreticulin (Basu *et al.*, 2001, Immunity 14(3):303-13).

Studies have shown that in addition to its proteinase-inhibitory functions, α2M, when complexed to antigens, can enhance the antigens' ability to be taken up by antigen presenting cells such as macrophages and presented to T cell hybridomas *in vitro* by up to two orders of magnitude (Chu and Pizzo, 1994, Lab. Invest. 71:792), and to induce T cell proliferation (Osada et al., 1987, Biochem. Biophys. Res. Commun.146:26-31). Further evidence suggests that complexing antigen with α2M enhances antibody production by crude spleen cells *in vitro* (Osada *et al.*, 1988, Biochem. Biophys. Res. Commun. 150:883), elicits an *in vivo* antibody responses in experimental rabbits (Chu *et al.*, 1994, J. Immunol. 152:1538-1545) and mice (Mitsuda *et al.*, 1993, Biochem. Biophys. Res. Commun. 101:1326-1331). α2M-antigenic peptide complexes have also been shown to induce a cytotoxic T cell response *in vivo* (Binder *et al.*, 2001, J. Immunol. 166:4698-49720).

## 3. SUMMARY OF THE INVENTION

The present invention encompasses the making and using of complexes of antigenic proteins and peptides and heat shock protein (HSP) or alpha-2-macroglobulin (α2M) for the prevention and treatment of cancer and infectious disease. Preferably, the complexes are used in combination with at least one non-heat shock protein and non-alpha-2-macroglobulin-based treatment modality.

In one embodiment, the invention uses complexes of HSPs and a population of antigenic proteins of antigenic cells or viral particles prepared by a method that involves complexing a population of antigenic proteins derived from antigenic cells or viral particles to one or more different heat shock proteins in vitro, wherein the population comprises at least 50% of the

different proteins or at least 50 different proteins that are present in the antigenic cells or viral particles, or present in a cellular fraction of the antigenic cells.

In another embodiment, the complexes are prepared by a method that comprises contacting the protein preparation in vitro with one or more different heat shock proteins under conditions such that proteins in the protein preparation are complexed to the heat shock proteins.

In yet another embodiment, the invention provides uses of complexes comprising HSPs and a population of antigenic peptides of antigenic cells or viral particles, wherein the population of antigenic peptides is generated by a method comprising digesting a protein preparation of antigenic cells, a cellular fraction thereof, or viral particles with either a protease or a plurality of different proteases separately. The population of antigenic peptides can also be generated by a method comprising exposing a protein preparation of antigenic cells, a cellular fraction thereof, or viral particles to ATP, guanidium hydrochloride, and/or acidic conditions sufficient to elute antigenic peptides from protein complexes present in the protein preparation. The antigenic peptides generated by either or both methods are complexed to one or more different HSPs in vitro.

In yet another embodiment, the invention provides uses of complexes of α2M and a population of antigenic proteins of antigenic cells. The complexes are prepared by a method that involves complexing a population of antigenic proteins derived from antigenic cells or viral particles to α2M in vitro, wherein the population comprises at least 50% of the different proteins or at least 50 different proteins that are present in the antigenic cells or viral particles, or present in a cellular fraction of the antigenic cells. In another embodiment, the method comprises contacting the protein preparation in vitro with α2M under conditions such that proteins in the protein preparation are complexed to α2M.

In yet another embodiment, the invention provides uses of complexes comprising α2M and a population of antigenic peptides of antigenic cells or viral particles, wherein the population of antigenic peptides is generated by a method comprising digesting a protein preparation of antigenic cells, a cellular fraction thereof, or viral particles, with either a protease or a plurality of different proteases separately. The population of antigenic peptides can also be generated by a method comprising exposing a protein preparation of antigenic cells, a cellular fraction thereof, or viral particles, with ATP, guanidium hydrochloride, and/or acidic conditions. The antigenic peptides generated by either or both methods are complexed to α2M in vitro.

In various embodiments, the antigenic cells can be cancer cells, or cells infected with a pathogen or infectious agent, and preferably human cells. The antigenic cells can also be cells of a pathogen or infectious agent, or variants thereof. The antigenic proteins/peptides can be prepared from cancer cells or cells infected with a pathogen that are antigenically related to the cancer or infectious diseases. A pathogen or infectious agent, including viral particles can also be used to prepare the antigenic peptides. The protein preparation of the antigenic cells may comprise only cytosolic proteins, only membrane-derived proteins, or both cytosolic and membrane-derived proteins. The protein preparation may be a crude, unfractionated cell lysate. In a specific embodiment, the protein preparation can be made by lysing the antigenic cells, removing cell debris and non-proteinaceous materials, and optionally purifying the proteins, by methods known in the art. In certain embodiments, the protein preparation has not been subjected to any method of preparation that selectively removes or retains one or more particular protein from the other proteins in the antigenic cells.

In certain embodiments, the protein preparation of the antigenic cells, a cellular fraction thereof, or viral particles can be digested by a variety of proteases, such as but not limited to trypsin, Staphylococcal peptidase I (also known as protease V8), chymotrypsin, pepsin, cathepsin G, thermolysin, elastase, and papain, under conditions suitable for enzymatic reaction. The extent of the digestion can be monitored by taking a sample and analyzing it by known techniques for determining the length of peptides. It is preferable that the digesting step is carried out under conditions such that the resulting population of peptides which comprises antigenic peptides, have an average size of from about 7 amino acid residues to about 20 amino acid residues. It is also desirable to generate from a protein preparation different populations of peptides by digesting aliquots of the protein preparation with different proteases. The peptides resulting from the different digests may be combined before complexing to HSP or α2M. Before complexing the population of peptides which comprises antigenic peptides to HSP or α2M, it may be desirable to inactivate or separate the protease from the population of peptides, and optionally purify the population of peptides.

In certain embodiments, the protein preparation of the antigenic cells, a cellular fraction thereof, or viral particles are contacted with adenosine triphosphate (ATP), guanidium hydrochloride, and/or acidic conditions such that antigenic peptides can be eluted without the need to isolate HSP complexes or α2M complexes initially. The antigenic peptides eluted by this

method comprise peptides that are endogenously associated with HSPs, α2M, and MHC class I and II molecules.

In various embodiments of the invention, depending on the method used to complex the population of antigenic peptides to HSP or α2M in vitro, the reaction can result in the antigenic peptides complexed to HSP or α2M by either a covalent bond or non-covalent bond. Heat shock proteins that are contemplated for complexing include but not limited to HSP 60, HSP70, HSP 90, gp96, calreticulin, grp78 (or BiP), protein disulfide isomerase (PDI), HSP110, and grp170. Human HSPs and human α2M are generally preferred. The complexes of HSP or α2M and antigenic peptides formed in vitro can be further purified before their use in or as a therapeutic or prophylactic composition. Such compositions may further comprises an adjuvant. Kits for combination therapy comprising HSP and/or α2M, antigenic cells, protein preparations, and/or proteases, and additional treatment modalities are also provided.

In another aspect, a method is provided for treating or preventing a type of cancer or infectious disease, comprising administering to a subject in need of such treatment or prevention (i) a composition comprising an amount, effective for said treatment or prevention, of HSP and/or α2M complexed to a population of antigenic peptides; and in combination with (ii) another treatment modality that is a non-HSP and non-α2M-based treatment modality. The additional treatment modality is preferably a non-vaccine treatment modality. Examples of treatment modalities include but are not limited to antibiotics, antivirals, antifungal compounds, antiprotozoal compounds, antihelminth compounds, anti-cancer treatments such as chemotherapeutic agents, antiangiogenic compounds, hormones, and radiation, as well as biological therapeutic agents and immunotherapeutic agents.

In another embodiment, a method is provided for treating or preventing a type of cancer or infectious disease, comprising administering to a subject in need of such treatment or prevention antigen presenting cells which have been sensitized with complexes of HSP and/or α2M and a population of antigenic proteins/peptides. In addition to the administration of sensitized antigen presenting cells to a subject, complexes of HSP and/or α2M and a population of antigenic peptides; and/or a non-HSP and non-α2M-based treatment modality can also be administered to the subject.

The invention also provides methods for improving the therapeutic outcome of a non-HSP and non-α2M-based treatment modality comprising administering either HSP complexes or

α2M complexes, preferably purified complexes, in conjunction with the administration of the treatment modality.

In one embodiment of the invention, a method is provided for inducing an immune response in a subject against a first antigenic cell or viral particle, wherein said subject is receiving a non-HSP and non-α2M treatment modality, said method comprising administering to the individual a composition comprising an immunogenic amount of HSP and/or α2M complexed to a population of antigenic proteins/peptides that were prepared from a second antigenic cell or viral particle. The antigenic peptides can be obtained by digesting the protein preparation of the antigenic cells or viral particles with a protease or exposing the protein preparation with ATP, guanidium hydrochloride and/or acidic condition. The first and second antigenic cells or viral particles express at least one common antigenic determinant.

In another embodiment, the present invention also provides a method for improving the outcome of a treatment in a subject receiving HSP complexes or α2M complexes, by administering another therapeutic modality to the subject before, concurrently with, or after the administration of the HSP complexes or α2M complexes. Either the HSP complexes or the α2M complexes can be administered over a period of time which may precede, overlap, and/or follow a treatment regimen with a non-vaccine treatment modality.

The administering of the HSP complexes or α2M complexes to a subject can be repeated at the same site, and periodically, for example, at weekly intervals. The composition can be administered by many routes, such as intradermally or subcutaneously.

In yet another embodiment, the invention encompasses methods of treatment that provide better therapeutic profiles than the administration of the treatment modality or the HSP complexes alone. In another embodiment, the invention encompasses methods of treatment that provide better therapeutic profiles than the administration of the treatment modality or the α2M complexes alone. Encompassed by the invention are methods wherein the administration of a treatment modality with an HSP complexes or an α2M complexes has additive potency or additive therapeutic effect. The invention also encompasses synergistic outcomes where the therapeutic efficacy is greater than additive. Preferably, such administration of a treatment modality with an HSP complexes or with an α2M complexes also reduces or avoids unwanted or adverse effects.

Given the invention, in certain embodiments, doses of non-vaccine treatment modality can be reduced or administered less frequently, in order to increase patient compliance, improve therapy and/or reduce unwanted or adverse effects. In a specific embodiment, lower or less frequent doses of chemotherapy or radiation therapy are administered to reduce or avoid unwanted effects. Alternatively, doses of HSP complexes and doses of α2M complexes can be reduced or administered less frequently if administered with a treatment modality. In certain embodiments, the administration of the HSP/α2M complexes in the absence of administration of the therapeutic modality or the administration of the therapeutic modality in the absence of administration of the HSP/α2M complexes is not therapeutically effective. In a specific embodiment, the amount of HSP/α2M complexes or therapeutic modality is administered in an amount insufficient to be therapeutically effective alone. In alternate embodiments, both or at least one of the HSP/α2M complexes or therapeutic modality is therapeutically effective when administered alone.

## 4. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for preparing and using a composition comprising heat shock protein (HSP) or alpha-2-macroglobulin (α2M) that are useful for the prevention and treatment of cancer and infectious disease. The methods of the invention comprise preparing in vitro complexes of HSP or α2M, and the antigenic proteins and peptides of antigenic cells and using it in combination with another treatment modality. In one embodiment, the method involves making a protein preparation of the antigenic cells which preparation comprises a population of antigenic proteins; and complexing in vitro the population of antigenic proteins to HSP or α2M. In another embodiment, the method further involves digesting the protein preparation of the antigenic cells with at least one protease to generate a population of antigenic peptides prior to complexing in vitro the population of antigenic peptides to HSP or α2M. The invention exploits the full antigenic potential of antigenic cells to generate a HSP- and/or α2M-based vaccine.

The therapeutic and prophylactic methods of the invention are based on eliciting an immune response in a subject in whom the treatment or prevention of infectious diseases or cancer is desired, and who has received or will receive another treatment modality. The immune response is directed specifically against antigenic determinants of cancerous cells, cells infected

by an infectious agent that causes the infectious disease, or antigenic determinants of the infectious agent. By administering to the individual a composition comprising a population of molecular complexes comprising HSPs and proteins/peptides of antigenic cells or a population of molecular complexes comprising α2M and proteins/peptides of antigenic cells, the molecular complexes in the composition can stimulate an immune response, such as a cytotoxic T cell response in the individual. The antigenic cells can be cancerous cells or infected cells, or cells which share antigenic determinants with or display similar antigenicity as the cancerous or infected cells. As a result of the immune response, various immune effector mechanisms of the individual will act on the cancerous or infected cells which can by itself or in combination with other treatment modalities lead to the treatment or prevention of such disease.

The individual or subject in whom treatment or prevention of an infectious diseases or cancer is desired is an animal, preferably a mammal, a non-human primate, and most preferably human. The term "animal" as used herein includes but is not limited to companion animals, such as cats and dogs; zoo animals; wild animals, including deers, foxes and racoons; farm animals, livestock and fowl, including horses, cattle, sheep, pigs, turkeys, ducks, and chickens, as well as any rodents.

The compositions and methods of the present invention are an improvement over other compositions and methods that use naturally-occurring HSP-antigenic peptide complexes to treat or prevent cancer or infectious disease. In such other methods, a specific HSP and its complexes with antigenic peptides are isolated from a cancer or infected cell, and administered to a patient to induce an immune response against the cancer or infected cells *in vivo* (*see e.g.*, U.S. Patent Nos. 5,750,119 and 5,961,979). Naturally-occurring complexes are isolated by methods dictated by the type of HSP which is desired. Thus, naturally-occurring complexes of a type of HSP and antigenic peptides comprise only those antigenic peptides that are co-localized in a compartment of the antigenic cells with that type of HSP. Certain types of HSPs are found uniquely in one cellular compartment and some antigenic peptides are found only in certain compartments of an antigenic cell. For example, HSP90 and HSP70 are found only in the cytosol. Thus, they will only be complexed to antigenic peptides located in the cytosol but not to antigenic peptides located somewhere else, such as the endoplasmic reticulum for example. That is, only a subset of the antigenic peptides of the antigenic cell can bind to each particular HSP. Thus, to stimulate an immune response to a maximum number of antigenic determinants of a cancer or infected cell,

every type of HSPs and their peptide complex would have to be isolated from the cancerous or infected cell by their respective methods of isolation, and then administered to a patient. This approach is laborious and may require large amounts of antigenic cells which is not available under certain circumstances. The methods of the present invention solve this problem by generating a peptide profile of virtually all the antigens of an antigenic cell *in vitro,* and then complexing the peptides to one or more different HSP and/or α2M which can then be used to stimulate an immune response in a patient. By using the methods of the invention, even antigenic peptides and HSPs that are not co-localized within the same compartment of an antigenic cell can form a complex. The methods of the invention afford the possibility to form complexes of a particular type of HSP with a majority of or even every antigenic peptides of an antigenic cell. Accordingly, a more effective immune response against antigenic cells can be induced by the compositions prepared by the methods of the invention. Moreover, this method does not require the prior isolation of HSP complexes and the associated peptides, thus, allowing the use of very small amount of starting material which is often limited in supply.

Moreover, the antigen profile of cancerous cells, infected cells, or pathogens may change over a period of time, e.g., even during a course of treatment. Many pathogens evade the host immune system by mutation and synthesis of mutant proteins that are not recognized by immune cells and antibodies. Cancerous cells are known to become resistant to certain drugs by mutations resulting in the synthesis of mutant proteins, some of which may not be recognized by the immune system. An advantage of using one of the embodiments of the present invention is that by digesting the cytosolic and/or membrane-derived proteins from cancerous cells, infected cells or pathogens, a wider range of antigenic proteins and hence a greater diversity of antigenic peptides are complexed to HSPs and/or α2M. As a result, the immune response is directed to a greater number of antigenic determinants on the antigenic cells, thus, making it more difficult for the antigenic cell, such as a cancer cell or an infected cell, to escape recognition and action by the immune system.

In another specific embodiment, the methods of the present invention generate α2M-peptide complexes that are not found naturally. α2M is an extracellular protein that is known to bind to various extracellular proteins, proteases in particular, to inactivate them and then bring them to the intracellular environment. α2M does not generally have access and therefore does not complex to the entire repertoire of antigenic peptides of an antigenic cell. The methods of the

present invention allow α2M to be complexed to a much wider range of peptides that are cytosolic or membrane-derived, or that are generated by the in vitro digestion of cytosolic and membrane-derived proteins of antigenic cells.

Described in Section 4.1 are sources of antigenic cells from which protein preparations can be made. In Section 4.2, methods for making different types of protein preparations of antigenic cells and methods for digesting a protein preparation are provided. Section 4.3 describes respectively the isolation or production of HSP or α2M, which are used in complexing with antigenic peptides. The in vitro complexing of HSP and antigenic peptides are described in Section 4.4. Described in Section 4.5 are methods of use of the complexes in the prevention and treatment of cancer and infectious agents, and the types of cancer and infectious diseases that are treated. The use of the compositions prepared by the methods of the invention in adoptive immunotherapy, is taught in Section 4.6. Section 5 provides experimental data showing the effectiveness of the complexes of the invention in protecting an animal prophylactically from cancer cell growth.

## 4.1. SOURCES OF ANTIGENIC CELLS

The antigenic cells of the invention comprise an antigenic determinant to which an immune response in a subject is desired.

For the treatment or prevention of cancer or infectious disease, the methods of the invention provide compositions of HSPs and α2M complexed to antigenic proteins and peptides, which antigenic proteins/peptides were derived from cancer cells, preferably human cancers, e.g., fragments of tumor-specific antigens and tumor associated antigens. The peptides can be generated by proteolytic digestion of proteins (e.g., cytosolic and/or membrane-derived proteins) from cancer cells, or antigenic cells that share antigenic determinants with or display similar antigenicity as the cancer cells. The antigenic peptides can also be generated by exposing the proteins to ATP, guanidium hydrochloride, and/or acidic conditions. As used herein, the term "cells or tissue of the same type of cancer" refers to cells or tissue of cancer of the same tissue type, or metastasized from cancer of the same tissue type.

For the treatment or prevention of infectious diseases, the methods of the invention provide compositions of HSPs and α2M complexed to antigenic peptides that were derived from cells infected by a pathogen or infectious agent that causes the infectious disease, or the pathogen

which includes but is not limited to, a virus, bacterium, fungus, protozoan, parasite, etc. Preferably, the pathogen is one that infects humans. The antigenic peptides are generated by proteolytic digestion of (e.g., cytosolic and/or membrane-derived) proteins obtained from infected cells, antigenic cells that share antigenic determinants with or display similar antigenicity as the infected cells, or the pathogens including viral particles. The antigenic peptides can also be generated by exposing the proteins to ATP, guanidium hydrochloride, and/or acid. The antigenic peptides can also be generated from antigenic cells that display the antigenicity of an agent (pathogen) that causes the infectious disease, or a variant of such agent.

Since whole cancer cells, infected cells or other antigenic cells are used in the present methods, it is not necessary to isolate or characterize or even know the identities of these antigenic peptides in advance of using the present methods. The source of the antigenic cells may be selected, depending on the nature of the disease with which the antigens are associated. In one embodiment of the invention, any tissues, or cells isolated from a cancer, including cancer that has metastasized to multiple sites, can be used as an antigenic cell in the present method. For example, leukemic cells circulating in blood, lymph or other body fluids can also be used, solid tumor tissue (e.g., primary tissue from a biopsy) can be used. As used herein, the term cancer cell also encompasses a preneoplastic cell which is a cell in transition from a normal to a neoplastic form. The transition from non-neoplastic cell growth to neoplasia commonly consists of hyperplasia, metaplasia, and dysplasia (for review of such abnormal growth conditions (*See* Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-79). A non-limiting list of cancers, the cells of which can be used herein is provided in Section 4.5.1 below.

In another embodiment of the invention, any cell that is infected with a pathogen or infectious agent, i.e., an infected cell, can be used as an antigenic cell for the preparation of antigenic peptides. In particular, cells infected by an intracellular pathogen, such as a virus, bacterium, fungus, parasite, or protozoan, is preferred. An exemplary list of infectious agents that can infect cells which can be used herein is provided in Section 4.5.2.

In yet another embodiment, any pathogen or infectious agent that can cause an infectious disease can be used as antigenic cell for the preparation of antigenic peptides. Variants of a pathogen or infectious agent, such as but limited to replication-defective variants, non-pathogenic or attenuated variants, non-infectious variants, can also be used as an antigenic cell for this

purpose. For example, many viruses, bacteria, fungi, parasites and protozoans that can be cultured in vitro or isolated from infected materials can serve as a source of antigenic cells. Methods known in the art for propagating such pathogens including viral particles can be used. An exemplary list of pathogens or infectious agents that can be used as antigenic cells is provided in Section 4.5.2.

Cell lines derived from cancer tissues, cancer cells, or infected cells can also be used as antigenic cells. Cancer or infected tissues, cells, or cell lines of human origin are preferred. Cancer cells, infected cells, or antigenic cells can be identified and isolated by any method known in the art. For example, cancer cells or infected cells can be identified by morphology, enzyme assays, proliferation assays, or the presence of pathogens or cancer-causing viruses. If the characteristics of the antigens of interest are known, antigenic cells can also be identified or isolated by any biochemical or immunological methods known in the art. For example, cancer cells or infected cells can be isolated by surgery, endoscopy, other biopsy techniques, isolation from body fluids (such as blood), affinity chromatography, and fluorescence activated cell sorting (e.g., with fluorescently tagged antibody against an antigen express by the cells). Antigenic cells that display similar antigenicity have one or more antigenic determinants in common against which an immune response in a subject is desired (e.g., for therapeutic or prophylactic purposes).

If the number of antigenic cells obtained from a subject is insufficient, the cells may be cultured in vitro by standard methods to expand the number of cells prior to use in the present methods. There is no requirement that a clonal or homogeneous or purified population of antigenic cells be used. A mixture of cells can be used provided that a substantial number of cells in the mixture contain the antigenic determinants or antigens of interest. In a specific embodiment, the antigenic cells and/or immune cells are purified.

In order to prepare pathogen-infected cells, uninfected cells of a cell type susceptible to infection by the pathogen or infectious agent that causes the disease can be infected in vitro. Depending on the mode of transmission and the biology of the pathogen or infectious agent, standard techniques can be used to facilitate infection by the pathogen or infectious agent, and propagation of the infected cells. For example, influenza viruses may be used to infect normal human fibroblasts; and mycobacteria may be used to infect normal human Schwann cells. In various embodiments, variants of an infectious agent, such as replication-defective viruses, non-pathogenic or attenuated mutants, or temperature-sensitive mutants can also be used to infect or

transform cells to generate antigenic cells for the preparation of antigenic peptides. If large numbers of a pathogen are needed to infect cells, or if pathogens are used directly as antigenic cells, any method known in the art can be used to propagate and grow the pathogens. Such methods will depend on the pathogen, and may not involve infecting a host. For example, many techniques are known in the art for growing pathogenic bacteria, fungi and other non-viral microorganisms in culture, including large scale fermentation.

Alternatively, if the gene encoding a tumor antigen (e.g., tumor-specific antigen and tumor-associated antigen) or antigen of the pathogen is available, normal cells of the appropriate cell type from the intended recipient may be transformed or transfected in vitro with an expression construct comprising a nucleic acid molecule encoding such antigen, such that the antigen is expressed in the recipient's cells. In one embodiment, a tumor-associated antigen is an antigen that is expressed at a higher level in a tumor cell relative to a normal cell; a tumor-specific antigen is an antigen that is expressed only in a tumor cell and not in a normal cell. Optionally, more than one such antigen may be expressed in the recipient's cell in this fashion, as will be appreciated by those skilled in the art, any techniques known, such as those described in Ausubel et al. (1989, Current Protocols in Molecular Biology, Wiley Interscience), may be used to perform the transformation or transfection and subsequent recombinant expression of the antigen gene in recipient's cells.

Suitable proteins and peptides that may be expressed in such cells include, but are not limited to those displaying the antigenicity of cancer cells. For example, such tumor specific or tumor-associated antigens include but are not limited to KS 1/4 pan-carcinoma antigen (Perez and Walker, 1990, J. Immunol. 142:3662-3667; Bumal, 1988, Hybridoma 7(4):407-415); ovarian carcinoma antigen (CA125) (Yu, *et al.*, 1991, Cancer Res. 51(2):468-475); prostatic acid phosphate (Tailer, *et al.*, 1990, Nucl. Acids Res. 18(16):4928); prostate specific antigen (Henttu and Vihko, 1989, Biochem. Biophys. Res. Comm. 160(2):903-910; Israeli, *et al.*, 1993, Cancer Res. 53:227-230); melanoma-associated antigen p97 (Estin, *et al.*, 1989, J. Natl. Cancer Inst. 81(6):445-446); melanoma antigen gp75 (Vijayasardahl, *et al.*, 1990, J. Exp. Med. 171(4):1375-1380); high molecular weight melanoma antigen (Natali, *et al.*, 1987, Cancer 59:55-63), prostate specific membrane antigen, tyrosinase, gp100, melan-A, and mucins. Other exogenous antigens that may be complexed to HSPs/α2M include portions or proteins that are mutated at a high frequency in cancer cells, such as oncogenes (e.g., ras, in particular mutants of

ras with activating mutations, which only occur in four amino acid residues (12, 13, 59 or 61) (Gedde-Dahl *et al.*, 1994, Eur. J. Immunol. 24(2):410-414)) and tumor suppressor genes (e.g., p53, for which a variety of mutant or polymorphic p53 peptide antigens capable of stimulating a cytotoxic T cell response have been identified (Gnjatic *et al.*, 1995, Eur. J. Immunol. 25(6):1638-1642).

Preferably, where it is desired to treat or prevent viral diseases, suitable proteins and peptides comprising epitopes of known viruses can be expressed in the appropriate cells. For example, such antigenic epitopes from viruses include, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsackie virus, mumps virus, measles virus, smallpox virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV-I), and human immunodeficiency virus type II (HIV-II).

Preferably, where it is desired to treat or prevent bacterial infections, suitable proteins and peptides comprising epitopes of known bacteria can be expressed in the appropriate cells. . For example, such bacterial epitopes may be derived from various bacteria including, but not limited to, Gram positive bacillus (e.g., *Listeria, Bacillus* such as *Bacillus anthracis*, *Erysipelothrix* species), Gram negative bacillus (e.g., *Bartonella, Brucella, Campylobacter, Enterobacter, Escherichia, Francisella, Hemophilus, Klebsiella, Morganella, Proteus, Providencia, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio,* and *Yersinia* species), spirochete bacteria (e.g., *Borrelia* species including *Borrelia burgdorferi* that causes Lyme disease, and *Leptospira*), anaerobic bacteria (e.g., *Actinomyces* and *Clostridium* species including *C. tetani, C. botulinum, C. perfringens*), Gram positive and negative coccal bacteria, *Streptococcus* species, *Pneumococcus* species, *Staphylococcus* species (e.g., *S. aureus* and *S. pneumonia*), *Neisseria* species (e.g., *N. meningitidis*).

Preferably, where it is desired to treat or prevent fungal infections, suitable proteins and peptides comprising epitopes of known fungi can be expressed in the appropriate cells. For example, such antigenic epitopes may be derived from various fungi including, *Aspergillus (e.g., Aspergillus fumigatus), Cryptococcus (e.g., Cryptococcus neoformans), Sporotrix, Coccidioides, Paracoccidioides, Histoplasma, Blastomyces, Candida (e.g., Candida albicans), Rhizopus, Rhizomucor, Absidia,* and *Basidiobolus* species.

Preferably, where it is desired to treat or prevent parasitic infections, suitable proteins and peptides comprising epitopes of known protozoa, nematodes, or helminths can be expressed in the appropriate cells. For example, such antigenic epitopes may be derived from various protozoa including, but not limited to, *Entoamoeba, Plasmodium, Leishmania, Eimeria, Cryptosporidium, Giardiasis, Toxoplasma,* and *Trypanosoma* species.

## 4.2. PREPARATION OF ANTIGENIC PROTEINS AND PEPTIDES

According to the invention, the compositions of the invention comprise antigenic proteins complexed to HSPs, wherein the antigenic proteins are from a preparation of proteins of the antigenic cells of interest. The compositions of the invention also comprise antigenic proteins complexed to α2M, wherein the antigenic proteins are from a preparation of proteins of the antigenic cells of interest. The compositions of the invention also comprise complexes of HSPs and antigenic peptides, or complexes of α2M and antigenic peptides that are prepared by first, generating a population of peptides from a preparation of proteins of the antigenic cells of interest, and then complexing the peptides to HSPs or α2M.

In various embodiments, to maximize and preserve the diversity of antigenic proteins and peptides, the methods used for preparing a protein preparation of antigenic cells do not selectively remove or retain any particular protein or peptide from other proteins and peptides in the antigenic cell. Even in certain embodiments when cytosolic proteins or membrane-derived proteins are used, the methods used to make the preparations do not selectively remove or retain any particular protein of the cytosol or of the membranes. Therefore, the majority of the proteins present in the cytosol or in the membranes are also present in the respective preparations of antigenic proteins and peptides from antigenic cells. In preferred embodiments, substantially the entire repertoire of antigenic proteins and peptides of the antigenic cells, and substantially all the antigenic proteins and peptides in the cytosol or in the membranes are present in the complexing reaction and form complexes with HSPs and/or α2M.

### 4.2.1 PROTEIN PREPARATIONS OF ANTIGENIC CELLS

In one embodiment of the invention, a protein preparation is provided which is derived from a cancer cell, infected cell, or pathogen. For example, for the treatment of cancer, the protein preparations are prepared, postoperatively, from tumor cells obtained from a cancer

patient. In another embodiment of the present invention, one or more antigenic proteins of interest are synthesized in cell lines modified by the introduction of recombinant expression systems that encode such antigens, and such cells are used to prepare the proteins. The proteins can be obtained from one or more cellular fraction(s), for example, the cytosol of the antigenic cells, or they can be extracted or solubilized from the membranes or cell walls of the antigenic cells. Any technique known in the art for cell lysis, fractionation of cellular contents, and protein enrichment or isolation can be used. See, for example, Current Protocols in Immunology, vol. 2, chapter 8, Coligan et al. (ed.), John Wiley & Sons, Inc.; Pathogenic and Clinical Microbiology: A Laboratory Manual by Rowland et al., Little Brown & Co., June 1994; which are incorporated herein by reference in their entireties. Depending on the techniques used to fractionate the cellular contents, a cellular fraction comprises at least 20, 50, 100, 500, 1,000, 5,000, 10,000, or 20,000 different proteins.

As used herein, the term "protein preparation" refers to a mixture of proteins obtained from antigenic cells, a cellular fraction of antigenic cells, or virus particles. The proteins can be obtained from a cellular fraction, such as the cytosol. The proteins can also be non-cytosolic proteins (e.g., those from cell walls, cell membranes or organelles), or both. Cellular fractions may include but are not limited to cytosolic fractions, membrane fractions, and organelle fractions, such as nuclear, mitochondrial, lysosomal, and endoplasmic reticulum-derived fractions. The protein preparations can be obtained from non-recombinant or recombinant cells. The term "antigenic proteins" as used herein also encompasses antigenic polypeptides and antigenic peptides that may be present in the preparation. The protein preparation obtained from the antigenic cells or cellular fractions thereof or virus particles can optionally be purified from other non-proteinaceous materials to various degrees by techniques known in the art. The protein preparation may comprise at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 97%, 98%, 99% of the different proteins and peptides present in the antigenic cells or virus particles or a fraction of the antigenic cells.

In a specific embodiment, the protein preparations have not been subjected to any method of preparation that selectively removes or retains one or more particular protein(s) from the other proteins in the antigenic cells.

In a specific embodiment, the protein preparation is the total cell lysate which is not fractionated and/or purified, and may contain other non-proteinaceous materials of the cells.

In another specific embodiment, the protein preparation is the total protein in a cellular fraction, which has not been subjected to further fractionation or purification, and may contain other non-proteinaceous materials of the cells.

In yet another embodiment, the protein preparation is the total protein in a preparation of viral particles.

In specific embodiments, the protein preparation comprises total cellular protein, total cytosolic proteins, or total membrane-bound proteins of antigenic cell(s).

In various embodiments, the protein preparation comprises at least 20, 50, 100, 500, 1,000, 5,000, 10,000, or 20,000 different proteins. A plurality of different antigenic proteins are present in a protein preparation of antigenic cells. Moreover, the proteins in the protein preparation may be subjected to a step of protease digestion prior to in vitro complexing to HSPs or α2M. Alternatively, the proteins in the protein preparation are not subjected to a step of protease digestion prior to in vitro complexing to HSPs or α2M.

To make a protein preparation of antigenic cells or virus particles, the lysing of antigenic cells or disruption of cell walls, cell membranes, or viral particle structure can be performed using standard protocols known in the art. In various embodiments, the antigenic cells can be lysed, for example, by mechanical shearing, sonication, freezing and thawing, adjusting the osmolarity of the medium surrounding the cells, or a combination of techniques. In less preferred embodiments, the antigenic cells can be lysed by chemicals, such as detergents.

Once the cells are lysed, it is desirable to remove cellular debris, materials that are non-proteinaceous or materials that do not contain cytosolic, and/or membrane-derived proteins (including proteins in the membranes of organelles). Removal of these components can be accomplished by techniques such as low speed centrifugation or filtration. After removing cellular debris and intact cells, a high speed centrifugation step can be used to separate the cytosolic proteins which are in the supernatant, and the membrane-derived proteins which are collected in the pellet. Standard procedures commonly known in the art allows the further isolation of the membrane-derived proteins from the pellet. Standard techniques commonly known in the art can be used to extract viral proteins from viral particles. These separation methods act on the basis of the general and overall size, density, and/or charge of the molecules that are present in the antigenic cell, in the cytosol or in the membranes. These separation

methods do not or are not designed to selectively remove or retain any one or more particular protein(s) from other proteins.

In various embodiments, the proteins from the antigenic cells can be optionally separated by their general biochemical and/or biophysical properties, such as size, density, charge, cellular location or combinations thereof. Many techniques known in the art can be used to perform the separation. Selected fractions of the proteins/peptides that comprise at least 20, 50, 100, 500, 1,000, 5,000, 10,000, or 20,000 different proteins or that comprise at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 97%, 98%, 99% of the different proteins present in the antigenic cells or a cellular fraction thereof, or virus particles, can be used to form complexes to HSP or α2M. Accordingly, the proteins from the antigenic cells can be prepared by methods that separate molecules by their size, charge, cellular location or a combination thereof, and that do not selectively remove or retain any one or more specific protein(s) from other proteins that are present in the antigenic cell, in the cytosol or in the membranes.

An exemplary, but not limiting, method that may be used to make a protein preparation comprising cytosolic proteins is as follows:

> Cells, which may be tumor cells derived from a biopsy of the patient or tumor cells cultivated *in vitro*, or cell infected with a pathogenic agent, are suspended in 3 volumes of 1X Lysis buffer comprising 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then the hypotonically-swollen cells are homogenized in a dounce homogenizer until >95% cells are lysed. As an alternative to shearing, cells can be sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. When sonication is used, cells are suspended in a buffer such as phosphate buffered saline (PBS) which may comprises 1 mM PMSF, before sonication.
>
> The lysate is centrifuged at 1,000 x g for 10 minutes to remove intact cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at about 100,000 x g for about one hour, and the supernatant recovered. The 100,000 x g supernatant may be dialyzed for 36 hours at 4°C (three times, 100 times volumes each time) against PBS or other suitable buffer, to provide the soluble cytosolic proteins of the present invention. If necessary, insoluble material in the preparation may be removed by filtration or low-speed centrifugation.

An exemplary, but not limiting, method that may be used to make a protein preparation comprising membrane-derived proteins is as follows:

Cells, which may be tumor cells derived from a biopsy of the patient or tumor cells cultivated *in vitro*, or cells infected with a pathogenic agent, are suspended in 3 volumes of 1X Lysis buffer comprising 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then the hypotonically-swollen cells are homogenized in a dounce homogenizer until >95% cells are lysed. As an alternative to shearing, cells can be sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. When sonication is used, cells are suspended in a buffer such as phosphate buffered saline (PBS) which may comprises 1 mM PMSF, before sonication.

The lysate is then centrifuged at 100,000 x g for 10 minutes to collect the cell membranes. Membrane-derived proteins can be dislodged from the lipid bilayer and isolated from the 100,000g pellet (where the membrane-derived proteins are located) by resuspending the pellet in 5 volumes of PBS containing 1% sodium deoxycholate (without $Ca^{2+}$ and $Mg^{2+}$) and incubated on ice for 1 h. The resulting suspension is centrifuged for 30 min at 20,000g and the resulting supernatant harvested and dialyzed against several changes of PBS (without $Ca^{2+}$ and $Mg^{2+}$) to remove the detergent. The resulting dialysate is centrifuged for 90 min at 100,000g and the supernatant purified further. Then calcium and magnesium are both added to the supernatant to give final concentrations of 2mM. If necessary, insoluble material in the preparation may be removed by filtration or low-speed centrifugation.

In a specific embodiment, the population of cytosolic and/or membrane-derived proteins obtained from antigenic cells can be complexed to HSP or α2M directly without protease treatment or any further extraction or selection processes. Alternatively, the proteins can be subjected to protease treatment prior to complexing.

### 4.2.2 PEPTIDES FROM ANTIGENIC CELLS

According to the invention, the cytosolic and membrane-derived proteins obtained from antigenic cells can be optionally digested to generate antigenic peptides. In one embodiment,

either the cytosolic or the membrane-derived proteins are used in the digestion. In another embodiment, the cytosolic and membrane-derived proteins are combined in the digestion reaction to generate antigenic peptides. In preferred embodiments, the protein preparations that are used in the protease digestion have not been subjected to any method(s) of preparation that selectively remove or retain one or more particular protein(s) from the other proteins in the antigenic cells, or the cytosol or membranes of the antigenic cells.

Various proteases or proteolytic enzymes can be used in the invention to produce from a protein preparation of antigenic cells a population of peptides which comprises antigenic peptides. The enzymatic digestions can be performed either individually or in suitable combinations with any of the proteolytic enzymes that are well known in the art including, but not limited to, trypsin, Staphylococcal peptidase I (also known as protease V8), chymotrypsin, pepsin, cathepsin G, thermolysin, elastase, and papain. Trypsin is a highly specific serine proteinase that cleaves on the carboxyl-terminal side of lysines and arginines. Due to the limited number of cleavage sites, it is expected to leave many MHC-binding epitopes intact. Staphylococcal peptidase I, a serine proteinase, has specificity for cleavage after glutamic and aspartic acid residues. A digestion can be carried out with a single protease or a mixture of proteases. The proteases or proteolytic enzymes used are incubated under conditions suitable for the particular enzyme. Preferably, the enzyme is purified. Non-enzymatic methods, such as cyanogen bromide cleavage, can also be used for generating peptides. The protein preparation to be digested can be aliquoted into a plurality of reactions each using a different enzyme, and the resulting peptides may optionally be pooled together for use. It may not be necessary to completely digest the proteins in the enzymatic reactions. These reactions results in the generation of a diverse and different set of peptides for each protein that is present in the protein preparation. The production of different peptide sets allows for a greater probability of generating antigenic peptides that are capable of inducing an immune response to the antigens in the protein preparation when they are complexed to HSP or α2M. In a preferred embodiment, the protein preparation to be digested is aliquoted into two separate reactions and two different proteolytic enzymes are used to produce two different sets of peptides of the proteins present in the protein preparation. Depending on the proteins, enzymes and reaction conditions, undigested proteins may remain in the reactions. In a preferred embodiment, trypsin and Staphylococcal peptidase I are used separately to digest the protein preparation.

In another preferred embodiment, the proteolytic enzymes used in the invention exhibit similar activities as the proteolytic activities that are found in the proteasome. The proteasome is responsible for the extralysosomal, endocatalytic degradation of cytosolic and nuclear proteins which are mis-folded or damaged in a cell. The proteasome can degrade proteins completely to yield single amino acids, can generate optimal major histocompatibility complex class I (MHC I)-binding epitopes, and can generate longer peptide precursors which could potentially undergo further trimming elsewhere in the cell to yield cytotoxic T cell epitopes. Cleavage preferences of the proteasome is on the carboxyl (COOH)-side of basic, acidic, and hydrophobic amino acids. Three known proteolytic enzymatic activities that are present in the proteasome are chymotrypsin-like activity, trypsin-like activity, and peptidylglutamylpeptide-hydrolyzing activity (Uebel and Tampe, 1999, Curr. Opin. Immunol. 11:2 203-208). As such, enzymes having such activities and specificities can be used separately or in combination to digest the protein preparation. In a preferred embodiment, trypsin, chymotrypsin, and/or peptidylglutamylpeptide-hydrolase are used.

The resulting peptide digestions comprise antigenic peptides, non-antigenic peptides, and single amino acid residues. The reactions may also comprise undigested or incompletely digested antigenic proteins. The proteolytic enzymatic digestions of the invention are monitored in order to generate peptides that fall within a desirable range of lengths. In a preferred embodiment, the peptides generated are from about 7 to about 20 amino acid residues. Most antigenic peptides that are presented to T cells by MHC class I and class II fall within this range. In various embodiments, the population of peptides comprises peptides having a size range of 6 to 21, 8 to 19, 10 to 20, or at least 7, 8, 9, 10, 11, 12, 15, 20, 25, 30, 40, 45, or 50, amino acid residues. In preferred embodiments, the antigenic peptides have 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues. To monitor the progression of protein digestion, a test reaction can be performed where small aliquots of a protein digestion are taken out of the reaction and monitored for the progression of digestion through either tricine-polyacrylamide gel electrophoresis ("tricine-PAGE"), high performance liquid chromatography ("HPLC"), or mass spectrometry, or any other method known in the art to determine the size of peptides. Using such a test reaction, a determination can be made as to when peptide fragments of a particular size range will be generated at a particular enzymatic concentration. Other variables of the reaction that can be

manipulated include the amount of protein in the reaction, the temperature, the duration of incubation, the presence of cofactors, etc.

Once the proper conditions are established for the generation of peptide fragments of a particular size range from a type of antigenic cell, the enzymatic reaction conditions can be duplicated to generate antigenic peptides which can be pooled. It is preferred that the enzymatic digestion is terminated before the peptides are complexed to HSPs or α2M. In one embodiment of the invention, inhibitors can be used for terminating an enzymatic digestion. Enzymatic inhibitors that can be used in the invention include, but are not limited to, PMSF, bestatin, amastatin, leupeptin, and cystatin, depending on which enzymes are used in the protein digestion. Inhibitors for most proteases are well known in the art. Alternatively, another method of terminating an enzymatic digestion is by physical removal of the enzyme from the reaction. This can be done by attaching the enzyme of choice to a solid phase, such as a resin or a material that can easily be removed from the reaction by well known methods such as centrifugation or filtration. The protein preparation is allowed to contact or flow across the solid phase for a period of time. Such immobilized enzymes can be purchased commercially or can be produced by procedures for immobilizing enzymes that are well known in the art.

At the end of the digestion reaction, the peptides can optionally be separated from low molecular weight materials, such as dipeptides, or single amino acid residues, in the preparation. For example, the peptides can be isolated by centrifugation through a membrane, such as the Centriprep-3. Optionally, the peptides can be separated by their general biochemical and/or biophysical properties, such as size, charge, or combinations thereof. Any techniques known in the art can be used to perform the separation resulting in digested protein preparation comprising at least 50, 100, 500, 1,000, 5,000, 10,000, 20,000, 50,000, or 100,000 different peptides.

In another embodiment of the invention, peptides that are endogenously present in antigenic cells can be used in the invention either alone or in combination with the peptides generated by the proteolytic digestion of the cytosolic and membrane-derived proteins. Peptides that are endogenously present in antigenic cells include peptides that are complexed in vivo to HSP and/or MHC class I and II molecules. According to the invention, such peptides that are isolated directly from a protein preparation of antigenic cells can be complexed to HSPs and/or α2M.

In specific embodiments, either the cytosolic or the membrane-derived proteins are used in the isolation process. In another specific embodiment, the cytosolic and membrane-derived proteins are combined in the isolation process. In preferred embodiments, the protein preparations that are used in the isolation have not been subjected to any method(s) of preparation that selectively remove or retain one or more particular protein(s) from the other proteins in the antigenic cells, or the cytosol or membranes of the antigenic cells. The antigenic peptides are isolated directly from a protein preparation of the cell without isolating complexes of antigenic peptides and HSP, α2M or major histocompatibility complex (MHC) molecules first. Preferably, the protein preparation comprises comprise at least 20, 50, 100, 500, 1,000, 5,000, 10,000, or 20,000 different proteins or that comprise at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 97%, 98%, 99% of the different proteins present in the antigenic cells or a cellular fraction thereof, or virus particles.

In various embodiments, the method comprise treating the protein preparation to ATP, guanidium hydrochloride, and/or exposing the protein preparation to acidic conditions such that antigenic peptides that are associated with proteins such as HSPs, α2M, and MHC class I and II molecules in the protein preparation can be eluted. Preferably, the isolation process does not comprise purifying HSP complexes, α2M complexes, or MHC complexes for the protein preparation prior to treatment with ATP, guanidium hydrochloride, or acidic conditions. Many different acids can be used, including but not limited to, trifluoroacetic acid. Methods are known in the art for the isolation of peptides from HSP-peptide complexes, such as Menoret *et al.*, 1999, Biochem. Biophys. Res. Commun. 262(3):813-8, which is incorporated herein by reference in its entirety. Methods known in the art such as those described in Marston and Hartley (1990, Meth. Enzymol. 182:264-276) for dissociating protein aggregates can also be used.

In particular, the isolation process comprises exposing a protein preparation of antigenic cells with ATP, for example, at room temperature for one hour, and/or treating a protein preparation of antigenic cells with trifluoroacetic acid (TFA) at a concentration in the range of 0.05% to 1% TFA. The treatment preferably comprises sonicating the protein preparation in the presence of 0.1% TFA. In a most preferred embodiment, a protein preparation is first exposed to ATP, followed by sonication in 0.1% TFA. Various protease inhibitors can be used in the invention prior to cell lysis and the isolation process to prevent or reduce cleavage of cellular protein that may generate peptides that are not endogenously associated with HSPs or or α2M.

For example, a mixture of 14 protease inhibitors can be used: phenylmethylsulfonyl fluoride (PMSF) 2 mM, ethylenediaminetetreacedic acid (EDTA) 1 mM, ethylene glycolbis(P-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) 1 mM, (all obtained from Sigma, St. Louis, MO), and Antipain 20 mg/ml, Bestatin 5 mg/ml, Chemostatin 20 ptg/nil, E64 20 Jig/ml, Leupeptine 1 ttg/ml, Pepstatine 1 gg/ml, Pefabloc 40 Ag/ml, and Apoprotein 10 tkg/rnl (all obtained from Boehringer Mannheim, Indianapolis, IN). The peptides resulting from the protein preparation comprise antigenic peptides and non-antigenic peptides of a variety of sizes ranging from at least 7, 8, 9, 10, 11, 12, 15, 20, 25, 30, 40, 45, or 50, amino acid residues. At the end of the process, the peptides are preferably recovered by separating from the proteins in the preparation prior to complexing with HSP or α2M. For example, the peptides can be recovered by centrifugation through a membrane, such as the Centriprep-3, by drying under vacuum, or by reverse phase chromatography, e.g., fractionation in a BioCad20 microanalytiocal HPLC Poros RH2 column (Perseptive Biosystems, Cambridge, MA), equilibrated with 0.1% TFA in water and elution by acetonitrile. Accordingly, antigenic peptides that are endogenously present in antigenic cells and that are isolated directly from a protein preparation can be complexed to HSPs and/or α2M. Alternatively, a mixed population of peptides comprising peptides that are endogenously present in antigenic cells and peptides from digested cytosolic and membrane-derived proteins, can be complexed to HSPs and/or α2M.

### 4.3. PREPARATION OF HSPs AND α2M

According to the present invention, antigenic peptides derived from antigenic cells are complexed to HSPs and/or α2M. Described herein are exemplary methods that can be used for isolating and preparing HSPs and α2M for use in the invention.

Heat shock proteins, which are also referred to interchangeably herein as stress proteins, useful in the practice of the instant invention can be selected from among any cellular protein that satisfies the following criteria. It is a protein whose intracellular concentration increases when a cell is exposed to a stressful stimuli, it is capable of binding other proteins or peptides, it is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or under acidic conditions; and it is a protein showing at least 35% homology with any cellular protein having the above properties.

The first stress proteins to be identified were the heat shock proteins (HSPs). As their name implies, HSPs are synthesized by a cell in response to heat shock. To date, five major classes of HSPs have been identified, based on the molecular weight of the family members. These classes are called sHSPs (small heat shock proteins), HSP60, HSP70, HSP90, and HSP100, where the numbers reflect the approximate molecular weight of the HSPs in kilodaltons. In addition to the major HSP families, an endoplasmic reticulum resident protein, calreticulin, has also been identified as yet another heat shock protein useful for eliciting an immune response when complexed to antigenic molecules (Basu and Srivastava, 1999, J. Exp. Med. 189:797-202). Other stress proteins that can be used in the invention include but are not limited to grp78 (or BiP), protein disulphide isomerase (PDI), HSP110, and grp170 ( Lin *et al.*, 1993, Mol. Biol. Cell, 4:1109-1119; Wang *et al.,* 2001, J. Immunol., 165:490-497). Many members of these families were found subsequently to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption, oxygen radicals, hypoxia and infection with intracellular pathogens. *(See* Welch, May 1993, *Scientific American* 56-64; Young, 1990, *Annu. Rev. Immunol.* 8:401-420; Craig, 1993, *Science* 260:1902-1903; Gething, *et al.*, 1992, *Nature* 355:33-45; and Lindquist, *et al.*, 1988, *Annu. Rev. Genetics* 22:631-677), the disclosures of which are incorporated herein by reference. It is contemplated that HSPs/stress proteins belonging to all of these families can be used in the practice of the instant invention.

The major HSPs can accumulate to very high levels in stressed cells, but they occur at low to moderate levels in cells that have not been stressed. For example, the highly inducible mammalian HSP70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon heat shock (Welch, *et al.*, 1985, *J. Cell. Biol.* 101:1198-1211). In contrast, HSP90 and HSP60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai, *et al.*, 1984, *Mol. Cell. Biol.* 4:2802-10; van Bergen en Henegouwen, *et al.*, 1987, *Genes Dev.* 1:525-31).

Heat shock proteins are among the most highly conserved proteins in existence. For example, DnaK, the HSP70 from E. coli has about 50% amino acid sequence identity with HSP70 proteins from excoriates (Bardwell, *et al.*, 1984, *Proc. Natl. Acad. Sci.* 81:848-852). The HSP60 and HSP90 families also show similarly high levels of intrafamilies conservation (Hickey, *et al.*, 1989, *Mol. Cell. Biol.* 9:2615-2626; Jindal, 1989, *Mol. Cell. Biol.* 9:2279-2283). In

addition, it has been discovered that the HSP60, HSP70 and HSP90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress. Therefore it is contemplated that the definition of heat shock protein or stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of the three families whose expression levels in a cell are enhanced in response to a stressful stimulus.

In an embodiment wherein the HSP portion of the HSP-antigenic peptide complex is desired to be purified from cells, exemplary purification procedures such as described in Sections 4.3.1- 4.3.3 below can be employed to purify HSP-peptide complexes, after which the HSPs can be separated from the endogenous HSP-peptide complexes in the presence of ATP or under acidic conditions, for subsequent *in vitro* complexing to a population of antigenic peptides. See Peng, et al., 1997, J. Immunol. Methods, 204:13-21; Li and Srivastava, 1993, EMBO J. 12:3143-3151, which are incorporated herein by reference in their activities. Although described for tumor cells, the protocols described hereinbelow may be used to isolate HSPs from any infected cells, and any eukaryotic cells, for example, tissues, isolated cells, or immortalized eukaryote cell lines infected with an intracellular pathogen, tumor cells or tumor cell lines.

### 4.3.1. PREPARATION AND PURIFICATION OF HSP70-PEPTIDE COMPLEXES

The purification of HSP70-peptide complexes has been described previously, see, for example, Udono *et al.*, 1993, *J. Exp. Med.* 178:1391-1396. A procedure that may be used, presented by way of example but not limitation, is described below.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 30mM sodium bicarbonate pH 7.5, 1mM PMSF. Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing by homogenizing the cells in a Dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with phosphate

buffered saline (PBS) containing 2mM $Ca^{2+}$ and 2mM $Mg^{2+}$. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1 mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated in 20mM Tris-Acetate pH 7.5, 20mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. The column is then developed with a 20mM to 500mM NaCl gradient and then eluted fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-HSP70 antibody (such as from clone N27F3-4, from StressGen).

Fractions strongly immunoreactive with the anti-HSP70 antibody are pooled and the HSP70-peptide complexes precipitated with ammonium sulfate; specifically with a 50%-70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex$^{R}$ G25 column (Pharmacia). If necessary the HSP70 preparation thus obtained can be repurified through the Mono Q FPLC Column as described above.

The HSP70-peptide complex can be purified to apparent homogeneity using this method. Typically 1 mg of HSP70-peptide complex can be purified from 1 g of cells/tissue.

An improved method for purification of HSP70 comprises contacting cellular proteins with ATP or a nonhydrolyzable analog of ATP affixed to a solid substrate, such that HSP70 in the lysate can bind to the ATP or nonhydrolyzable ATP analog, and eluting the bound HSP70. A preferred method uses column chromatography with ATP affixed to a solid substratum (*e.g.*, ATP-agarose). The resulting HSP70 preparations are higher in purity and devoid of contaminating peptides. The HSP70 yields are also increased significantly by about more than 10 fold.

Alternatively, chromatography with nonhydrolyzable analogs of ADP, instead of ATP, can be used for purification of HSP70-peptide complexes. By way of example but not limitation,

purification of HSP70 free of peptide by ATP-agarose chromatography can be carried out as follows:

Meth A sarcoma cells (500 million cells) are homogenized in hypotonic buffer and the lysate is centrifuged at 100,000 g for 90 minutes at 4°C. The supernatant is applied to an ATP-agarose column. The column is washed in buffer and is eluted with 5 column volumes of 3 mM ATP. The HSP70 elutes in fractions 2 through 10 of the total 15 fractions which elute. The eluted fractions are analyzed by SDS-PAGE. The HSP70 can be purified to apparent homogeneity using this procedure.

### 4.3.2. PREPARATION AND PURIFICATION OF HSP90-PEPTIDE COMPLEXES

A procedure that can be used, presented by way of example but not limitation, is described below.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 30mM sodium bicarbonate pH 7.5, 1mM PMSF. Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing by homogenizing the cells in a Dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with PBS containing 2mM $Ca^{2+}$ and 2mM $Mg^{2+}$. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 20mM sodium phosphate pH 7.4, 1 mM EDTA, 250mM NaCl. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated with dialysis buffer. The proteins are then eluted with a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and fractions containing the HSP90-peptide complexes identified by immunoblotting using an anti-HSP90 antibody such as 3G3 (Affinity Bioreagents). HSP90-peptide complexes can be purified to apparent homogeneity using

this procedure. Typically, 150-200 μg of HSP90-peptide complex can be purified from 1g of cells/tissue.

### 4.3.3. PREPARATION AND PURIFICATION OF GP96-PEPTIDE COMPLEXES

A procedure that can be used, presented by way of example but not limitation, is described below.

A pellet of tumors is resuspended in 3 volumes of buffer consisting of 30mM sodium bicarbonate buffer (pH 7.5) and 1mM PMSF and the cells allowed to swell on ice 20 minutes. The cell pellet is then homogenized in a Dounce homogenizer (the appropriate clearance of the homogenizer will vary according to each cell type) on ice until >95% cells are lysed.

The lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step is then recentrifuged at 100,000g for 90 minutes. The gp96-peptide complex can be purified either from the 100,000 pellet or from the supernatant.

When purified from the supernatant, the supernatant is diluted with equal volume of 2X lysis buffer and the supernatant mixed for 2-3 hours at 4°C with Con A Sepharose equilibrated with PBS containing 2mM $Ca^{2+}$ and 2mM $Mg^{2+}$. Then, the slurry is packed into a column and washed with 1X lysis buffer until the $OD_{280}$ drops to baseline. Then, the column is washed with 1/3 column bed volume of 10% α-methyl mannoside (α-MM) dissolved in PBS containing 2mM $Ca^{2+}$ and 2mM $Mg^{2+}$, the column sealed with a piece of parafilm, and incubated at 37°C for 15 minutes. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the α-MM buffer are applied to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60-95% pure, however this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q FPLC column (Pharmacia) equilibrated with a buffer containing 5mM sodium phosphate, pH 7. The proteins are then eluted from the column with a 0-1M NaCl gradient and the gp96 fraction elutes between 400mM and 550mM NaCl.

The procedure, however, may be modified by two additional steps, used either alone or in combination, to consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and

the other optional step involves DEAE-Sepharose purification after the Con A purification step but before the Mono Q FPLC step.

In the first optional step, described by way of example as follows, the supernatant resulting from the 100,000g centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition of ammonium sulfate. The ammonium sulfate is added slowly while gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred from about ½ to 12 hours at 4°C and the resulting solution centrifuged at 6,000 rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2mM $Ca^{2+}$ and $Mg^{2+}$. Undissolved material is removed by a brief centrifugation at 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose and the procedure followed as before.

In the second optional step, described by way of example as follows, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5mM sodium phosphate buffer, pH 7, 300mM NaCl by dialysis, or preferably by buffer exchange on a Sephadex G25 column. After buffer exchange, the solution is mixed with DEAE-Sepharose previously equilibrated with 5mM sodium phosphate buffer, pH 7, 300mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured into a column. Then, the column is washed with 5mM sodium phosphate buffer, pH 7, 300mM NaCl, until the absorbance at 280nm drops to baseline. Then, the bound protein is eluted from the column with five volumes of 5mM sodium phosphate buffer, pH 7, 700mM NaCl. Protein containing fractions are pooled and diluted with 5mM sodium phosphate buffer, pH 7 in order to lower the salt concentration to 175mM. The resulting material then is applied to the Mono Q FPLC column (Pharmacia) equilibrated with 5mM sodium phosphate buffer, pH 7 and the protein that binds to the Mono Q FPLC column (Pharmacia) is eluted as described before.

It is appreciated, however, that one skilled in the art may assess, by routine experimentation, the benefit of incorporating the second optional step into the purification protocol. In addition, it is appreciated also that the benefit of adding each of the optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000g pellet, the pellet is suspended in 5 volumes of PBS containing either 1% sodium deoxycholate or 1% oxtyl glucopyranoside (but without the $Mg^{2+}$ and $Ca^{2+}$) and incubated on ice for 1 hour. The suspension is centrifuged at 20,000g for 30 minutes and the resulting supernatant dialyzed against several changes of PBS (also without the $Mg^{2+}$ and $Ca^{2+}$) to remove the detergent. The dialysate is centrifuged at 100,000g for 90 minutes, the supernatant harvested, and calcium and magnesium are added to the supernatant to give final concentrations of 2mM, respectively. Then the sample is purified by either the unmodified or the modified method for isolating gp96-peptide complex from the 100,000g supernatant, see above.

The gp96-peptide complexes can be purified to apparent homogeneity using this procedure. About 10-20μg of gp96 can be isolated from 1g cells/tissue.

### 4.3.4. PREPARATION AND PURIFICATION OF α2M

Alpha-2-macroglobulin can be bought from commercial sources or prepared by purifying it from human blood.

Generally, alpha-2-macroglobulin can be recovered and purified from sera of mammals by known methods, including ammonium sulfate precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, immunoaffinity chromatography, hydroxyapatite chromatography, and lectin chromatography.

In one embodiment, α2M are purified from serum using affinity purification techniques. Methods for chromatography fractionation of proteins, such as affinity chromatography, are well known in the art. Briefly, affinity chromatography utilizes an immobilized binding partner to specifically capture the protein in the binding reaction. The binding partner molecule of the affinity capture assay can comprise, for example, an antibody to α2M or other ligand, such as an α2M receptor binding domain which specifically binds α2M. Alternatively, a filter binding assay utilizes a device, such as a solid phase surface such as a filter or a column, to non-specifically retain proteins or protein complexes based on some physical or chemical difference between the complexes and the unbound reactants. Affinity chromatography and/or filter binding separation techniques may be used to isolate α2M from serum or other bodily fluid as described herein.

In a specific embodiment of the invention, α2M are isolated from serum as follows: serum is contacted to a solid phase, such as an agarose column, which contains a binding partner of α2M, i.e., an α2M- binding molecule. The serum is allowed to incubate on the solid phase for a period of time sufficient to allow binding of α2M with the solid phase. The material which does not bind is then removed from the solid phase; and the bound α2M is eluted from the solid phase.

The binding partner of α2M may be any molecule which specifically binds to α2M. In a preferred embodiment, the α2M - binding molecule is an antibody specific to α2M. The α2M - specific antibody is preferably a monoclonal antibody. In another preferred embodiment, the α2M - binding molecule is a ligand-binding fragment of the α2M receptor.

The solid phase may be any surface or matrix, such as, but not limited to, polycarbonate, polystyrene, polypropylene, polyethylene, glass, nitrocellulose, dextran, nylon, polyacrylamide and agarose. The support configuration can include beads, membranes, microparticles, the interior surface of a reaction vessel such as a microtiter plate, test tube or other reaction vessel.

In a preferred embodiment, α2M are isolated from serum from mice by diluting serum 1:1 with 0.04 M Tris pH 7.6, 0.15 M NaCl. The mixture is then applied to a 65ml Sephacryl S 300R (Sigma) column equilibrated and eluted with the same buffer. α2M-positive fractions are determined by dot blot and the buffer changed to a 0.01 M sodium phosphate buffer at pH 7.5 by use of a PD-10 column. Alternatively, the 0.04 M Tris pH 7.6, 0.15 M NaCl buffer can be used as buffer in ht e65ml column to eliminate the step of exchanging the buffer. The complex-containing fractions are applied to a Concanavalin A sepharose column. Bound complex are eluted with 0.2M methylmannose pyranoside, or 5% methylmannose pyranoside, and applied to a DEAE column equilibrated with 0.05M sodium acetate buffer. A2M are eluted in a pure form, as analyzed by SDS-PAGE and immunoblotting with 0.13 M sodium acetate buffer.

In yet another embodiment, α2M can be isolated from blood, the following non-limiting protocol can be used by way of example: blood is collected from a subject and is allowed to clot. It is then centrifuged for 30 minutes under 14,000 x g to obtain the serum which is then applied to a gel filtration column (Sephacryl S-300R) equilibrated with 0.04M Tris buffer pH 7.6 plus 0.3M NaCl. A 65ml column is used for about 10ml of serum. Three ml fractions are collected and each fraction is tested for the presence of α2M by dot blot using an α2M specific antibody. The α2M positive fractions are pooled and applied to a PD10 column to exchange the buffer to

.01M Sodium Phosphate buffer pH 7.5 with PMSF. The pooled fractions are then applied to a Con A column (10ml) equilbrated with the phosphate buffer. The column is washed and the protein is eluted with 5% methylmannose pyranoside. The eluent is passed over a PD10 column to change the buffer to a Sodium Acetate buffer (0.05M; pH6.0). A DEAE column is then equilibrated with acetate buffer and the sample is applied to the DEAE column. The column is washed and the protein is eluted with 0.13M sodium acetate. The fractions with α2M are then pooled. The α2M can be purified to apparent homogeneity using this procedure as assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Other methods for isolation of α2M known in the art can also be used (Dubin et al., 1984, Immunotherapy 8(4):589-596,; Okubo et al., 1981, Bio. Chem. Biophys. 688:257-267; Nieuwenhuizen et al. 1979, Biochem. Et Biophy. 580:129-139).

### 4.3.5. PREPARATION AND PURIFICATION OF NONCOVALENT CELLULARLY PRODUCED HSP110-PEPTIDE COMPLEXES

A procedure, described by Wang *et al.*, 2001, J. Immunol. 166(1):490-7, that can be used, presented by way of example and not limitation, is as follows:

A pellet (40-60 ml) of cell or tissue, *e.g.*, tumor cell tissue, is homogenized in 5 vol of hypotonic buffer (30 mN sodium bicarbonate, pH7.2, and protease inhibitors) by Dounce homogenization. The lysate is centrifuged at 4,500 × g and then 100,000 × g for 2 hours. If the cells or tissues are of hepatic origin, the resulting supernatant is was first applied to a blue Sepharose column (Pharmacia) to remove albumin. Otherwise, the resulting supernatant is applied to a Con A-Sepharose column (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with binding buffer (20mM Tris-HCl, pH 7.5; 100mM NaCl; 1mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM $MnCl_2$; and 15 mM 2-ME). The bound proteins are eluted with binding buffer containing 15% α-D-o-methylmannoside (Sigma, St. Louis, MO).

Con A-Sepharose unbound material is first dialyzed against a solution of 20 mM Tris-HCl, pH 7.5; 100 mM NaCl; and 15 mM 2-ME, and then applied to a DEAE-Sepharose column and eluted by salt gradient from 100 to 500 mM NaCl. Fractions containing hsp110 are collected, dialyzed, and loaded onto a Mono Q (Pharmacia) 10/10 column equilibrated with 20mM Tris-HCl, pH 7.5; 200 mM NaCl; and 15 mM 2-ME. The bound proteins are eluted with

a 200-500 mM NaCl gradient. Fractions are analyzed by SDS-PAGE followed by immunoblotting with an Ab for hsp110, as described by Wang *et al.*, 1999, J. Immunol. 162:3378. Pooled fractions containing hsp110 are concentrated by Centriplus (Amicon, Beverly, MA) and applied to a Superose 12 column (Pharmacia). Proteins are eluted by 40 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 15 mM 2-ME with a flow rate of 0.2 ml/min.

### 4.3.6. PREPARATION AND PURIFICATION OF NONCOVALENT CELLULARLY PRODUCED GRP170-PEPTIDE COMPLEXES

A procedure, described by Wang *et al.*, 2001, J. Immunol. 166(1):490-7, that can be used, presented by way of example and not limitation, is as follows:

A pellet (40-60 ml) of cell or tissue, *e.g.*, tumor cell tissue, is homogenized in 5 vol of hypotonic buffer (30 mN sodium bicarbonate, pH7.2, and protease inhibitors) by Dounce homogenization. The lysate is centrifuged at 4,500 × g and then 100,000 × g for 2 hours. If the cells or tissues are of hepatic origin, the resulting supernatant is was first applied to a blue Sepharose column (Pharmacia) to remove albumin. Otherwise, the resulting supernatant is applied to a Con A-Sepharose column (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with binding buffer (20mM Tris-HCl, pH 7.5; 100mM NaCl; 1mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM $MnCl_2$; and 15 mM 2-ME). The bound proteins are eluted with binding buffer containing 15% α-D-o-methylmannoside (Sigma, St. Louis, MO).

Con A-Sepharose-bound material is first dialyzed against 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl and then applied to a Mono Q column and eluted by a 150 to 400 mM NaCl gradient. Pooled fractions are concentrated and applied on the Superose 12 column (Pharmacia). Fractions containing homogeneous grp170 are collected.

### 4.3.7. RECOMBINANT EXPRESSION OF HEAT SHOCK PROTEINS AND α2M

In certain embodiments of the present invention, HSPs and α2M can be prepared from cells that express higher levels of HSPs and α2M through recombinant means. Amino acid sequences and nucleotide sequences of many HSPs and α2M are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession

number. These databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. Such nucleotide sequences of non-limiting examples of HSPs that can be used for the compositions, methods, and for preparation of the HSP peptide-complexes of the invention are as follows: human HSP70, Genbank Accession No. M24743, Hunt *et al.*, 1995, Proc. Natl. Acad. Sci. U.S.A., 82: 6455-6489; human HSP90, Genbank Accession No. X15183, Yamazaki *et al.*, Nucl. Acids Res. 17: 7108; human gp96: Genbank Accession No. X15187, Maki *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 5658-5562; human BiP: Genbank Accession No. M19645; Ting *et al.*, 1988, DNA 7: 275-286; human HSP27, Genbank Accession No. M24743; Hickey *et al.*, 1986, Nucleic Acids Res. 14: 4127-45; mouse HSP70: Genbank Accession No. M35021, Hunt *et al.*, 1990, Gene 87: 199-204; mouse gp96: Genbank Accession No. M16370, Srivastava *et al.*, 1987, Proc. Natl. Acad. Sci. U.S.A. 85: 3807-3811; and mouse BiP: Genbank Accession No. U16277, Haas *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. 85: 2250-2254. Degenerate sequences encoding HSPs can also be used.

As used herein, the term "α2M" embraces other polypeptide fragments, analogs, and variants of α2M having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with α2M, and is capable of forming a complex with an antigenic peptide, which complex is capable of being taken up by an antigen presenting cell and eliciting an immune response against the antigenic molecule. The α2M molecule of the invention can be purchased commercially or purified from natural sources (Kurecki *et al.*, 1979, Anal. Biochem. 99:415-420), chemically synthesized, or recombinantly produced. Non-limiting examples of α2M sequences that can be used for preparation of the α2M polypeptides of the invention are as follows: Genbank Accession Nos. M11313, P01023, AAA51551; Kan *et al.*, 1985, Proc. Nat. Acad. Sci. 82: 2282-2286. A degenerate sequence encoding α2M can also be used.

Once the nucleotide sequence encoding the HSP or α2M of choice has been identified, the nucleotide sequence, or a fragment thereof, can be obtained and cloned into an expression vector for recombinant expression. The expression vector can then be introduced into a host cell for propagation of the HSP or α2M. Methods for recombinant production of HSPs or α2M are described in detail herein.

The DNA may be obtained by DNA amplification or molecular cloning directly from a tissue, cell culture, or cloned DNA (*e.g.*, a DNA "library") using standard molecular

biology techniques (see *e.g.*, Methods in Enzymology, 1987, volume 154, Academic Press; Sambrook *et al.* 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Current Protocols in Molecular Biology, Ausubel et al. (eds.), Greene Publishing Associates and Wiley Interscience, New York, each of which is incorporated herein by reference in its entirety). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the HSP or α2M gene should be cloned into a suitable vector for propagation of the gene.

In a preferred embodiment, DNA can be amplified from genomic or cDNA by polymerase chain reaction (PCR) amplification using primers designed from the known sequence of a related or homologous HSP or α2M. PCR is used to amplify the desired sequence in DNA clone or a genomic or cDNA library, prior to selection. PCR can be carried out, *e.g.*, by use of a thermal cycler and Taq polymerase (Gene Amp®). The polymerase chain reaction (PCR) is commonly used for obtaining genes or gene fragments of interest. For example, a nucleotide sequence encoding an HSP or α2M of any desired length can be generated using PCR primers that flank the nucleotide sequence encoding open reading fram. Alternatively, an HSP or α2M gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) if such sites are available, releasing a fragment of DNA encoding the HSP or α2M gene. If convenient restriction sites are not available, they may be created in the appropriate positions by site-directed mutagenesis and/or DNA amplification methods known in the art (see, for example, Shankarappa *et al.*, 1992, PCR Method Appl. 1: 277-278). The DNA fragment that encodes the HSP or α2M is then isolated, and ligated into an appropriate expression vector, care being taken to ensure that the proper translation reading frame is maintained.

In an alternative embodiment, for the molecular cloning of an HSP or α2M gene from genomic DNA, DNA fragments are generated to form a genomic library. Since some of the sequences encoding related HSPs or α2M are available and can be purified and labeled, the cloned DNA fragments in the genomic DNA library may be screened by nucleic acid hybridization to a labeled probe (Benton and Davis, 1977, Science 196: 180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72: 3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify an appropriate fragment by

restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map.

Alternatives to isolating the HSP or α2M genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or synthesizing a cDNA to the mRNA which encodes the HSP or α2M. For example, RNA for cDNA cloning of the HSP or α2M gene can be isolated from cells which express the HSP or α2M. A cDNA library may be generated by methods known in the art and screened by methods, such as those disclosed for screening a genomic DNA library. If an antibody to the HSP or α2M is available, the HSP or α2M may be identified by binding of a labeled antibody to the HSP- or α2M-synthesizing clones.

Other specific embodiments for the cloning of a nucleotide sequence encoding an HSP or α2M, are presented as examples but not by way of limitation, as follows: In a specific embodiment, nucleotide sequences encoding an HSP or α2M can be identified and obtained by hybridization with a probe comprising a nucleotide sequence encoding HSP or α2M under various conditions of stringency which are well known in the art (including those employed for cross-species hybridizations).

Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, or for creating/deleting restriction sites to facilitate further manipulations. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson *et al.*, 1978, J. Biol. Chem. 253: 6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19: 423-463; Hill *et al.*, 1987, Methods Enzymol. 155: 558-568), PCR-based overlap extension (Ho *et al.*, 1989, Gene 77: 51-59), PCR-based megaprimer mutagenesis (Sarkar *et al.*, 1990, Biotechniques 8: 404-407), *etc.* Modifications can be confirmed by double stranded dideoxynucleotide DNA sequencing.

In certain embodiments, a nucleic acid encoding a secretory form of a non-secreted HSP is used to practice the methods of the present invention. Such a nucleic acid can be constructed by deleting the coding sequence for the ER retention signal, KDEL. Optionally, the KDEL coding sequence is replaced with a molecular tag to facilitate the recognition and purification of the HSP, such as the Fc portion of murine IgG1. In another embodiment, a molecular tag can be added to naturally secreted HSPs or α2M. PCT publication no. WO

99/42121 demonstrates that deletion of the ER retention signal of gp96 resulted in the secretion of gp96-Ig peptide-complexes from transfected tumor cells, and the fusion of the KDEL-deleted gp96 with murine IgG1 facilitated its detection by ELISA and FACS analysis and its purification by affinity chromatography with the aid of Protein A.

### 4.3.7.1 EXPRESSION SYSTEMS

Nucleotide sequences encoding an HSP or α2M molecule can be inserted into the expression vector for propagation and expression in recombinant cells. An expression construct, as used herein, refers to a nucleotide sequence encoding an HSP or α2M operably associated with one or more regulatory regions which allows expression of the HSP or α2M molecule in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the HSP or α2M polypeptide sequence to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation of the HSP or α2M sequence. A variety of expression vectors may be used for the expression of HSPs or α2M, including, but not limited to, plasmids, cosmids, phage, phagemids, or modified viruses. Examples include bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). Typically, such expression vectors comprise a functional origin of replication for propagation of the vector in an appropriate host cell, one or more restriction endonuclease sites for insertion of the HSP or α2M gene sequence, and one or more selection markers.

For expression of HSPs or α2M in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the β-interferon gene, and the HSP70 gene (Williams *et al.*, 1989, Cancer Res. 49: 2735-42 ; Taylor *et al.*, 1990, Mol. Cell. Biol. 10: 165-75). The efficiency of expression of the HSP or α2M in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes,

metallothionein, β-actin (see Bittner *et al.*, 1987, Methods in Enzymol. 153: 516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1: 36-47).

The expression vector may also contain sequences that permit maintenance and replication of the vector in more than one type of host cell, or integration of the vector into the host chromosome. Such sequences may include but are not limited to replication origins, autonomously replicating sequences (ARS), centromere DNA, and telomere DNA. It may also be advantageous to use shuttle vectors that can be replicated and maintained in at least two types of host cells.

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating or identifying host cells that contain DNA encoding an HSP or α2M. For long term, high yield production of HSPs or α2M, stable expression in mammalian cells is preferred. A number of selection systems may be used for mammalian cells, including, but not limited, to the Herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. U.S.A. 48: 2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22: 817) genes can be employed in *tk*⁻, *hgprt*⁻ or *aprt*⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (*dhfr*), which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. U.S.A. 77: 3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150: 1); and hygromycin phosphotransferase (hyg), which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30: 147). Other selectable markers, such as but not limited to histidinol and Zeocin™ can also be used.

The expression construct comprising an HSP- or α2M-coding sequence operably associated with regulatory regions can be directly introduced into appropriate host cells for expression and production of the HSP or α2M complexes of the invention without further cloning (see, for example, U.S. Patent No. 5,580,859). The expression constructs may also contain DNA sequences that facilitate integration of the coding sequence into the genome of the host cell, *e.g.*, via homologous recombination. In this instance, it is not necessary to employ an

expression vector comprising a replication origin suitable for appropriate host cells in order to propagate and express the HSP or α2M molecule in the host cells.

Expression constructs containing cloned HSP or α2M coding sequences can be introduced into the mammalian host cell by a variety of techniques known in the art, including but not limited to calcium phosphate mediated transfection (Wigler *et al.*, 1977, Cell 11: 223-232), liposome-mediated transfection (Schaefer-Ridder *et al.*, 1982, Science 215: 166-168), electroporation (Wolff *et al.*, 1987, Proc. Natl. Acad. Sci. 84: 3344), and microinjection (Cappechi, 1980, Cell 22: 479-488).

Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences by techniques well known in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are described in Appendix 5 of Current Protocols in Molecular Biology, 1988, ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, which is incorporated herein by reference; and the catalogs of commercial suppliers such as Clontech Laboratories, Stratagene Inc., and Invitrogen, Inc.

Alternatively, number of viral-based expression systems may also be utilized with mammalian cells for recombinant expression of HSPs or α2M. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer *et al.*, 1979, Cell 17: 725), adenovirus (Van Doren *et al.*, 1984, Mol. Cell Biol. 4: 1653), adeno-associated virus (McLaughlin *et al.*, 1988, J. Virol. 62: 1963), and bovine papillomas virus (Zinn *et al.*, 1982, Proc. Natl. Acad. Sci. 79: 4897). In cases where an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control region, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts (see, *e.g.*, Logan and Shenk, 1984, Proc. Natl. Acad. Sci. U.S.A. 81: 3655-3659).

Bovine papillomavirus (BPV) can infect many higher vertebrates, including man, and its DNA replicates as an episome. A number of shuttle vectors have been developed for recombinant gene expression which exist as stable, multicopy (20-300 copies/cell)

extrachromosomal elements in mammalian cells. Typically, these vectors contain a segment of BPV DNA (the entire genome or a 69% transforming fragment), a promoter with a broad host range, a polyadenylation signal, splice signals, a selectable marker, and "poisonless" plasmid sequences that allow the vector to be propagated in *E. coli*. Following construction and amplification in bacteria, the expression gene construct is transfected into cultured mammalian cells, for example, by the techniques of calcium phosphate coprecipitation or electroporation. For those host cells that do not manifest a transformed phenotype, selection of transformants is achieved by use of a dominant selectable marker, such as histidinol and G418 resistance. For example, BPV vectors such as pBCMGSNeo and pBCMGHis may be used to express HSPs or α2M (Karasuyama *et al.*, Eur. J. Immunol. 18: 97-104; Ohe *et al.*, Human Gene Therapy 6: 325-33) which may then be transfected into a diverse range of cell types for HSP or α2M expression.

Alternatively, the vaccinia 7.5K promoter may be used (see, e.g., Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A. 79: 7415-7419; Mackett *et al.*, 1984, J. Virol. 49: 857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A. 79: 4927-4931) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) may be used. Such vectors can be used with a broad range of human host cells, e.g., EBO-pCD (Spickofsky *et al.*, 1990, DNA Prot. Eng. Tech. 2: 14-18), pDR2 and λDR2 (available from Clontech Laboratories).

Recombinant HSP or α2M expression can also be achieved by a retrovirus-based expression system. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. In retroviruses such as Moloney murine leukemia virus, most of the viral gene sequences can be removed and replaced with an HSP or α2M coding sequence, while the missing viral functions can be supplied in *trans*. The host range for infection by a retroviral vector can also be manipulated by the choice of envelope used for vector packaging.

For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The ND-associated antigenic peptide DNA is inserted into a position between the 5' LTR and 3' LTR, such that transcription from the 5' LTR promoter transcribes the cloned DNA. The 5' LTR comprises a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the

art. A heterologous promoter as well as multiple drug selection markers may also be included in the expression vector to facilitate selection of infected cells (see McLauchlin *et al.*, 1990, Prog. Nucleic Acid Res. and Molec. Biol. 38: 91-135; Morgenstern *et al.*, 1990, Nucleic Acid Res. 18: 3587-3596; Choulika *et al.*, 1996, J. Virol 70: 1792-1798; Boesen *et al.*, 1994, Biotherapy 6: 291-302; Salmons and Gunzberg, 1993, Human Gene Therapy 4: 129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3: 110-114).

The recombinant cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition. Alternatively, cells may be cultured under conditions emulating the nutritional and physiological requirements of a cell in which the HSP is endogenously expressed. Modified culture conditions and media may be used to enhance production of HSP–peptide complexes. For example, recombinant cells may be grown under conditions that promote inducible HSP expression.

Alpha-2-macroglobulin and HSP polypeptides of the invention may be expressed as fusion proteins to facilitate recovery and purification from the cells in which they are expressed. For example, an HSP or α2M polypeptide may contain a signal sequence leader peptide to direct its translocation across the ER membrane for secretion into culture medium. Further, an HSP or α2M polypeptide may contain an affinity label, such as a affinity label, fused to any portion of the HSP or α2M polypeptide not involved in binding antigenic peptide, such as for example, the carboxyl terminal. The affinity label can be used to facilitate purification of the protein, by binding to an affinity partner molecule.

Various methods for production of such fusion proteins are well known in the art. The manipulations which result in their production can occur at the gene or protein level, preferably at the gene level. For example, the cloned coding region of an HSP or α2M polypeptide may be modified by any of numerous recombinant DNA methods known in the art (Sambrook *et al.*, 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Ausubel *et al.*, in Chapter 8 of Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York). It will be apparent from the following discussion that substitutions, deletions, insertions, or any combination thereof are introduced or combined to arrive at a final nucleotide sequence encoding an HSP or α2M polypeptide.

In various embodiments, fusion proteins comprising the HSP or α2M polypeptide may be made using recombinant DNA techniques. For example, a recombinant gene encoding an HSP or α2M polypeptide may be constructed by introducing an HSP or α2M gene fragment in the proper reading frame into a vector containing the sequence of an affinity label, such that the HSP or α2M polypeptide is expressed as a peptide-tagged fusion protein. Affinity labels, which may be recognized by specific binding partners, may be used for affinity purification of the HSP or α2M polypeptide.

In a preferred embodiment, the affinity label is fused at its amino terminal to the carboxyl terminal of HSP or α2M. The precise site at which the fusion is made in the carboxyl terminal is not critical. The optimal site can be determined by routine experimentation.

A variety of affinity labels known in the art may be used, such as, but not limited to, the immunoglobulin constant regions, polyhistidine sequence (Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan *et al.*, 1987, Gene 67:21-30), and various cellulose binding domains (U.S. Patent Nos. 5,496,934; 5,202,247; 5,137,819; Tomme *et al.*, 1994, Protein Eng. 7:117-123), etc. Other affinity labels may impart fluorescent properties to an HSP or α2M polypeptide, *e.g.*, portions of green fluorescent protein and the like. Other possible affinity labels are short amino acid sequences to which monoclonal antibodies are available, such as but not limited to the following well known examples, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope. Other affinity labels are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner which can be immobilized onto a solid support. Some affinity labels may afford the HSP or α2M polypeptide novel structural properties, such as the ability to form multimers. Dimerization of an HSP or α2M polypeptide with a bound peptide may increase avidity of interaction between the HSP or α2M polypeptide and its partner in the course of antigen presentation. These affinity labels are usually derived from proteins that normally exist as homopolymers. Affinity labels such as the extracellular domains of CD8 (Shiue *et al.*, 1988, J. Exp. Med. 168:1993-2005), or CD28 (Lee *et al.*, 1990, J. Immunol. 145:344-352), or portions of the immunoglobulin molecule containing sites for interchain disulfide bonds, could lead to the formation of multimers. As will be appreciated by those skilled in the art, many methods can be

used to obtain the coding region of the above-mentioned affinity labels, including but not limited to, DNA cloning, DNA amplification, and synthetic methods. Some of the affinity labels and reagents for their detection and isolation are available commercially.

A preferred affinity label is a non-variable portion of the immunoglobulin molecule. Typically, such portions comprise at least a functionally operative CH2 and CH3 domain of the constant region of an immunoglobulin heavy chain. Fusions are also made using the carboxyl terminus of the Fc portion of a constant domain, or a region immediately amino-terminal to the CH1 of the heavy or light chain. Suitable immunoglobulin-based affinity label may be obtained from IgG-1, -2, -3, or -4 subtypes, IgA, IgE, IgD, or IgM, but preferably IgG1. Preferably, a human immunoglobulin is used when the HSP or α2M polypeptide is intended for *in vivo* use for humans. Many DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries. See, for example, Adams *et al.*, Biochemistry, 1980, 19:2711-2719; Gough *et al.*, 1980, Biochemistry, 19:2702-2710; Dolby *et al.*, 1980, Proc. Natl. Acad. Sci. U.S.A., 77:6027-6031; Rice *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A., 79:7862-7865; Falkner *et al.*, 1982, Nature, 298:286-288; and Morrison *et al.*, 1984, Ann. Rev. Immunol, 2:239-256. Because many immunological reagents and labeling systems are available for the detection of immunoglobulins, the HSP or α2M polypeptide–Ig fusion protein can readily be detected and quantified by a variety of immunological techniques known in the art, such as the use of enzyme-linked immunosorbent assay (ELISA), immunoprecipitation, fluorescence activated cell sorting (FACS), etc. Similarly, if the affinity label is an epitope with readily available antibodies, such reagents can be used with the techniques mentioned above to detect, quantitate, and isolate the HSP or α2M polypeptide containing the affinity label. In many instances, there is no need to develop specific antibodies to the HSP or α2M polypeptide.

A particularly preferred embodiment is a fusion of an HSP or α2M polypeptide to the hinge, the CH2 and CH3 domains of human immunoglobulin G-1 (IgG-1; see Bowen *et al.*,1996, J. Immunol. 156:442-49). This hinge region contains three cysteine residues which are normally involved in disulfide bonding with other cysteines in the Ig molecule. Since none of the cysteines are required for the peptide to function as a tag, one or more of these cysteine residues may optionally be substituted by another amino acid residue, such as for example, serine.

Various leader sequences known in the art can be used for the efficient secretion of HSP or α2M polypeptide from bacterial and mammalian cells (von Heijne, 1985, J. Mol. Biol. 184:99-

105). Leader peptides are selected based on the intended host cell, and may include bacterial, yeast, viral, animal, and mammalian sequences. For example, the herpes virus glycoprotein D leader peptide is suitable for use in a variety of mammalian cells. A preferred leader peptide for use in mammalian cells can be obtained from the V-J2-C region of the mouse immunoglobulin kappa chain (Bernard *et al.*, 1981, Proc. Natl. Acad. Sci. 78:5812-5816). Preferred leader sequences for targeting HSP or α2M polypeptide expression in bacterial cells include, but are not limited to, the leader sequences of the *E.coli* proteins OmpA (Hobom *et al.*, 1995, Dev. Biol. Stand. 84:255-262), Pho A (Oka *et al.*, 1985, Proc. Natl. Acad. Sci 82:7212-16), OmpT (Johnson *et al.*, 1996, Protein Expression 7:104-113), LamB and OmpF (Hoffman & Wright, 1985, Proc. Natl. Acad. Sci. USA 82:5107-5111), β-lactamase (Kadonaga *et al.*, 1984, J. Biol. Chem. 259:2149-54), enterotoxins (Morioka-Fujimoto *et al.*, 1991, J. Biol. Chem. 266:1728-32), and the *Staphylococcus aureus* protein A (Abrahmsen *et al.*, 1986, Nucleic Acids Res. 14:7487-7500), and the *B. subtilis* endoglucanase (Lo *et al.*, Appl. Environ. Microbiol. 54:2287-2292), as well as artificial and synthetic signal sequences (MacIntyre *et al.*, 1990, Mol. Gen. Genet. 221:466-74; Kaiser *et al.*, 1987, Science, 235:312-317).

DNA sequences encoding a desired affinity label or leader peptide, which may be readily obtained from libraries, produced synthetically, or may be available from commercial suppliers, are suitable for the practice of this invention. Such methods are well known in the art.

### 4.4. COMPLEXING PROTEINS AND PEPTIDES TO HSP AND α2M

Described herein are exemplary methods for complexing in vitro the HSP or α2M with a population of proteins and/or peptides which have been prepared from antigenic cells, a cellular fraction thereof, or viral particles. The population of proteins and/or peptides are from a protein preparation of the antigenic cells as described in Section 4.2.1. In certain embodiments, the peptides are the result of digestion of a protein preparation of antigenic cells, a cellular fraction thereof, or viral particles. The complexing reaction can result in the formation of a covalent bond between a HSP and a protein or peptide of the antigenic cell or viral particle. The complexing reaction can result in the formation of a covalent bond between a α2M and a protein or peptide of the antigenic cell or viral particle. The complexing reaction can also result in the formation of a non-covalent association between a HSP and a protein and/or a peptide, or a α2M and a protein and/or a peptide.

Prior to complexing, the HSPs can be pretreated with ATP or exposed to acidic conditions to remove any peptides that may be non-covalently associated with the HSP of interest. When the ATP procedure is used, excess ATP is removed from the preparation by the addition of apyranase as described by Levy, *et al.*, 1991, *Cell* 67:265-274. When acidic conditions are used, the buffer is readjusted to neutral pH by the addition of pH modifying reagents. A preferred, exemplary protocol for the noncovalent complexing of a population of peptides (average length between 7 to 20 amino acids) to an HSP *in vitro* is discussed below:

The population of peptides (1μg, which can be dissolved in 10% to 50% dimethyl sulfoxide) and the pretreated HSP (9μg) are admixed to give an approximately 5 peptides (or proteins) : 1 HSP molar ratio. Then, the mixture is incubated for 15 minutes to 3 hours at 4° to 45°C in a suitable binding buffer such as phosphate buffered saline pH7.4, or one containing 20mM sodium phosphate, pH 7.2, 350mM NaCl, 3mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). The preparations are centrifuged through a Centricon 10 assembly (Millipore) to remove any unbound peptide. The non-covalent association of the proteins/peptides with the HSPs can be assayed by High Performance Liquid Chromatography (HPLC) or Mass Spectrometry (MS).

In an alternative embodiment of the invention, preferred for producing non-covalent complexes of HSP70 to proteins/peptides, 5-10 micrograms of purified HSP70 is incubated with equimolar quantities of proteins/peptides in 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3mM $MgCl_2$ and 1mM ADP in a volume of 100 microliter at 37°C for 1 hr. This incubation mixture is centrifuged one or more times if necessary, through a Centricon 10 assembly (Millipore) to remove any unbound peptide.

In an alternative embodiment of the invention, preferred for producing non-covalent complexes of gp96 or HSP90 to peptides, 5-10 micrograms of purified gp96 or HSP90 is incubated with equimolar or excess quantities of the proteins/peptides in a suitable buffer such as one containing 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3mM MgCl2 at 60-65°C for 5-20 min. This incubation mixture is allowed to cool to room temperature and centrifuged one or more times if necessary, through a Centricon 10 assembly (Millipore) to remove any unbound peptide.

Following complexing with antigenic proteins and/or antigenic peptides, an immunogenic HSP complex or α2M complex can optionally be assayed using, for example, the mixed

lymphocyte target cell assay (MLTC) described below. Once HSP-peptide complexes and/or HSP-protein complexes have been isolated and diluted, they can be optionally characterized further in animal models using the preferred administration protocols and excipients discussed below.

As an alternative to making non-covalent complexes of HSPs and proteins/peptides, a population of proteins/peptides can be covalently attached to HSPs.

In one embodiment, HSPs are covalently coupled to proteins and/or peptides in a protein preparation by chemical crosslinking. Chemical crosslinking methods are well known in the art. For example, in a preferred embodiment, glutaraldehyde crosslinking may be used. Glutaradehyde crosslinking has been used for formation of covalent complexes of peptides and HSPs (see Barrios *et al.*, 1992, Eur. J. Immunol. 22: 1365-1372). Preferably, 1-2 mg of HSP-peptide complex is crosslinked in the presence of 0.002% glutaraldehyde for 2 hours. Glutaraldehyde is removed by dialysis against phosphate buffered saline (PBS) overnight (Lussow *et al.*, 1991, Eur. J. Immunol. 21: 2297-2302). Alternatively, a HSP and a population of protein/peptides can be crosslinked by ultraviolet (UV) crosslinking under conditions known in the art.

In another embodiment of the invention, a population of proteins and/or peptides in a protein preparation can be non-covalently complexed to α2M by incubating the proteins/peptides with α2M at a 50:1 molar ratio and incubated at 50° C for 10 minutes followed by a 30 minute incubation at 25°C. Free (uncomplexed) peptides can be removed by size exclusion filters. Complexes are preferably measured by a scintillation counter to make sure that on a per molar basis, each HSP or α2M is observed to bind equivalent amounts of proteins/peptide (approximately 0.1% of the starting amount of the peptide). For details, see Binder, 2001, J. Immunol. 166(8):4968-72, which is incorporated herein by reference in its entirety. To reduce the propensity of forming covalent complexes of α2M and the proteins and peptides in these reactions, it will be desirable to inhibit or remove protease activity prior to complexing. This can be accomplished with the use of protease inhibitors, for example, by the methods described in section 4.2.1. Also desirable is adding a reducing agent (such as 2-mercaptoethanol) to the reactions to neutralize nucleophilic compounds present in the protein preparation which may activate α2M for covalent association.

In yet another embodiment, a population of antigenic proteins and/or antigenic peptides in a protein preparation can be complexed to α2M covalently by methods as described in PCT publications WO 94/14976 and WO 99/50303 for complexing a peptide to α2M, which are incorporated herein by reference in their entirety. For example, antigenic proteins and/or antigenic peptides can be incorporated into α2M by ammonia or methylamine (or other small amine nucleophiles such as ethylamine) during reversal of the nucleophilic activation, employing heat (Grøn and Pizzo, 1998, Biochemistry, 37: 6009-6014; which is incorporated herein by reference in its entirety). Such conditions that allow fortuitous trapping of peptides by α2M can be employed to prepare the α2M complexes of the invention. Covalent linking of a population of antigenic proteins/peptides to α2M can also be performed using a bifunctional crosslinking agent. Such crosslinking agents and methods of their use are also well known in the art. Preferably, the crosslinking agent is inactivated and/or removed after the complexes are formed. Methods for covalent coupling have been described previously (Osada *et al.*, 1987, Biochem. Biophys. Res. Commun.146:26-31; Osada *et al.*, 1988, Biochem. Biophys. Res. Commun. 150:883; Chu and Pizzo, 1993, J. Immunol. 150:48; Chu *et al.*, 1994, Ann. N.Y. Acad. Sci. 737:291-307; Mitsuda *et al.*, 1993, Biochem. Biophys. Res. Commun. 101:1326-1331).

In yet another embodiment, a population of proteins/peptides can be complexed to a mixture of HSP and α2M in the same reaction by the non-covalent or covalent methods described above.

Complexes of HSP and antigenic proteins and/or peptides from separate covalent and/or non-covalent complexing reactions can optionally be combined to form a composition before administration to a subject. Complexes of α2M and antigenic proteins and/or peptides from separate covalent and/or non-covalent complexing reactions can also optionally be combined to form a composition before administration to a subject.

## 4.5. PREVENTION AND TREATMENT OF CANCER AND INFECTIOUS DISEASES

In accordance with the invention, a composition of the invention, which comprises complexes of antigenic peptides derived from digested cytosolic and/or membrane-derived proteins of antigenic cells or viral particle and a HSP and/or α2M, is administered to a subject with cancer or an infectious disease. In one embodiment, "treatment" or "treating" refers to an amelioration of cancer or an infectious disease, or at least one discernible symptom thereof. In

another embodiment, "treatment" or "treating" refers to an amelioration of at least one measurable physical parameter associated with cancer or an infectious disease, not necessarily discernible by the subject. In yet another embodiment, "treatment" or "treating" refers to inhibiting the progression of a cancer or an infectious disease, either physically, *e.g.*, stabilization of a discernible symptom, physiologically, *e.g.*, stabilization of a physical parameter, or both.

In certain embodiments, the compositions of the present invention are administered to a subject as a preventative measure against such cancer or an infectious disease. As used herein, "prevention" or "preventing" refers to a reduction of the risk of acquiring a given cancer or infectious disease. In one mode of the embodiment, the compositions of the present invention are administered as a preventative measure to a subject having a genetic predisposition to a cancer. In another mode of the embodiment, the compositions of the present invention are administered as a preventive measure to a subject facing exposure to carcinogens including but not limited to chemicals and/or radiation, or to a subject facing exposure to an agent of an infectious disease.

For example, in certain embodiments, administration of the compositions of the invention leads to an inhibition or reduction of the growth of cancerous cells or infectious agents by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the growth in absence of said composition.

The compositions prepared by methods of the invention comprise complexes of heat shock protein(s) with a population of antigenic peptides, and/or complexes of alpha-2-macroglobulin with a population of antigenic peptides. The compositions appear to induce an inflammatory reaction at the tumor site and can ultimately cause a regression of the tumor burden in the cancer patients treated. The compositions prepared by the methods of the invention can enhance the immunocompetence of the subject and elicit specific immunity against infectious agents or specific immunity against preneoplastic and neoplastic cells. These compositions have the capacity to prevent the onset and progression of infectious diseases, and to inhibit the growth and progression of tumor cells.

Combination therapy refers to the use of HSP complexes or α2M complexes of the invention with another modality to prevent or treat cancer and infectious diseases. The administration of the complexes of the invention can augment the effect of anti-cancer agents or anti-infectives, and vice versa. Preferably, this additional form of modality is a non-HSP and

non-α2M based modality, i.e., this modality does not comprise either HSP or α2M as a component. This approach is commonly termed combination therapy, adjunctive therapy or conjunctive therapy (the terms are used interchangeably herein). With combination therapy, additive potency or additive therapeutic effect can be observed. Synergistic outcomes where the therapeutic efficacy is greater than additive can also be expected. The use of combination therapy can also provide better therapeutic profiles than the administration of the treatment modality, or the HSP complexes or α2M complexes alone. The additive or synergistic effect may allow the dosage and/or dosing frequency of either or both modalities be adjusted to reduce or avoid unwanted or adverse effects.

In various specific embodiments, the combination therapy comprises the administration of HSP complexes or α2M complexes to a subject treated with a treatment modality wherein the treatment modality administered alone is not clinically adequate to treat the subject such that the subject needs additional effective therapy, *e.g.*, a subject is unresponsive to a treatment modality without administering HSP complexes or α2M complexes. Included in such embodiments are methods comprising administering HSP complexes or α2M complexes to a subject receiving a treatment modality wherein said subject has responded to therapy yet suffers from side effects, relapse, develops resistance, etc. Such a subject might be non-responsive or refractory to treatment with the treatment modality alone, *i.e.*, at least some significant portion of cancer cells or pathogens are not killed or their cell division is not arrested. The embodiments provide that the methods of the invention comprising administration of HSP complexes to a subject refractory to a treatment modality alone can improve the therapeutic effectiveness of the treatment modality when administered as contemplated by the methods of the invention. The methods of the invention comprising administration of an α2M complexes to a subject refractory to a treatment modality alone can also improve the therapeutic effectiveness of the treatment modality when administered as contemplated by the methods of the invention. The determination of the effectiveness of a treatment modality can be assayed *in vivo* or *in vitro* using methods known in the art. Art-accepted meanings of refractory are well known in the context of cancer. In one embodiment, a cancer or infectious disease is refractory or non-responsive where respectively, the number of cancer cells or pathogens has not been significantly reduced, or has increased. Among these subjects being treated are those receiving chemotherapy or radiation therapy.

According to the invention, complexes of the invention can be used in combination with many different types of treatment modalities. Some of such modalities are particularly useful for a specific type of cancer or infectious disease and are discussed in Section 4.5.1 and 4.5.2. Many other modalities have an effect on the functioning of the immune system and are applicable generally to both neoplastic and infectious diseases .

In one embodiment, complexes of the invention are used in combination with one or more biological response modifiers to treat cancer or infectious disease. One group of biological response modifiers is the cytokines. In one such embodiment, a cytokine is administered to a subject receiving HSP/α2M complexes. In another such embodiment, HSP/α2M complexes are administered to a subject receiving a chemotherapeutic agent in combination with a cytokine. In various embodiments, one or more cytokine(s) can be used and are selected from the group consisting of IL-1α, IL- 1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IFNα, IFNβ, IFNγ, TNFα, TNFβ, G-CSF, GM-CSF, TGF-β, IL-15, IL-18, GM-CSF, INF-γ, INF-α, SLC, endothelial monocyte activating protein-2 (EMAP2), MIP-3α, MIP-3β, or an MHC gene, such as HLA-B7. Addtionally, other exemplary cytokines include other members of the TNF family, including but not limited to TNF-α-related apoptosis-inducing ligand (TRAIL), TNF-α-related activation-induced cytokine (TRANCE), TNF-α-related weak inducer of apoptosis (TWEAK), CD40 ligand (CD40L), lymphotoxin alpha (LT-α), lymphotoxin beta (LT-β), OX40 ligand (OX40L), Fas ligand (FasL), CD27 ligand (CD27L), CD30 ligand (CD30L), 41BB ligand (41BBL), APRIL, LIGHT, TL1, TNFSF16, TNFSF17, and AITR-L, or a functional portion thereof. See, *e.g.*, Kwon *et al.*, 1999, Curr. Opin. Immunol. 11:340-345 for a general review of the TNF family. Preferably, the HSP complexes or α2M complexes is administered prior to the treatment modalities. In a specific embodiment, complexes of the invention are administered to a subject receiving cyclophosphamide in combination with IL-12 for treatment of cancer.

In another embodiments, complexes of the invention are used in combination with one or more biological response modifiers which are agonists or antagonists of various ligands, receptors and signal transduction molecules of the immune system. For examples, the biological response modifiers include but are not limited to agoinsts of Toll-like receptors (TLR-2, TLR-7, TLR-8 and TLR-9; LPS; agonists of 41BB ligand, OX40 ligand, ICOS, and CD40; and

antagonists of Fas ligand, PD1, and CTLA-4. These agonists and antagonists can be antibodies, antibody fragments, peptides, peptidomimetic compounds, and polysaccharides.

In yet another embodiment, complexes of the invention are used in combination with one or more biological response modifiers which are immunostimulatory nucleic acids. Such nucleic acids, many of which are oligonucleotides comprising an unmethylated CpG motif, are mitogenic to vertebrate lymphocytes, and are known to enhance the immune response. See Woolridge, *et al.*, 1997, *Blood* 89:2994-2998. Such oligonucleotides are described in International Patent Publication Nos. WO 01/22972, WO 01/51083, WO 98/40100 and WO 99/61056, each of which is incorporated herein in its entirety, as well as United States Patent Nos. 6,207,646, 6,194,388, 6,218,371, 6,239,116, 6,429,199, and 6,406,705, each of which is incorporated herein in its entirety. Other kinds of immunostimulatory oligonucleotides such as phosphorothioate oligodeoxynucleotides containing YpG- and CpR-motifs have been described by Kandimalla *et al.* in "Effect of Chemical Modifications of Cytosine and Guanine in a CpG-Motif of Oligonucleotides: Structure-Immunostimulatory Activity Relationships." Bioorganic & Medicinal Chemistry 9:807-813 (2001), incorporated herein by reference in its entirety. Also encompassed are immunostimulatory oligonucleotides that lack CpG dinucleotides which when administered by mucosal routes (including low dose administration) or at high doses through parenteral routes, augment antibody responses, often as much as did the CpG nucleic acids, however the response was Th2-biased (IgG1>>IgG2a). See United States Patent Publication No. 20010044416 A1, which is incorporated herein by reference in its entirety. Methods of determining the activity of immunostimulatory oligonucleotides can be performed as described in the aforementioned patents and publications. Moreover, immunostimulatory oligonucleotides can be modified within the phosphate backbone, sugar, nucleobase and internucleotide linkages in order to modulate the activity. Such modifications are known to those of skill in the art.

In yet another embodiment, complexes of the invention are used in combination with one or more adjuvants. The adjuvant(s) can be administered separately or present in a composition in admixture with complexes of the invention. A systemic adjuvant is an adjuvant that can be delivered parenterally. Systemic adjuvants include adjuvants that creates a depot effect, adjuvants that stimulate the immune system and adjuvants that do both. An adjuvant that creates a depot effect as used herein is an adjuvant that causes the antigen to be slowly released in the body, thus prolonging the exposure of immune cells to the antigen. This class of adjuvants includes but is

not limited to alum (e.g., aluminum hydroxide, aluminum phosphate); or emulsion-based formulations including mineral oil, non-mineral oil, water-in-oil or oil-in-water-in oil emulsion, oil-in-water emulsions such as Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720, AirLiquide, Paris, France); MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, Calif.; and PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micelle-forming agent; IDEC, Pharmaceuticals Corporation, San Diego, Calif.).

Other adjuvants stimulate the immune system, for instance, cause an immune cell to produce and secrete cytokines or IgG. This class of adjuvants includes but is not limited to immunostimulatory nucleic acids, such as CpG oligonucleotides; saponins purified from the bark of the Q. saponaria tree, such as QS21; poly[di(carboxylatophen- oxy)phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides (LPS) such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, Mont.), muramyl dipeptide (MDP; Ribi) andthreonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmania elongation factor (a purified Leishmania protein; Corixa Corporation, Seattle, Wash.).

Other systemic adjuvants are adjuvants that create a depot effect and stimulate the immune system. These compounds are those compounds which have both of the above-identified functions of systemic adjuvants. This class of adjuvants includes but is not limited to ISCOMs (Immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia); SB-AS2 (SmithKline Beecham adjuvant system #2 which is an oil-in-water emulsion containing MPL and QS21: SmithKline Beecham Biologicals [SBB], Rixensart, Belgium); SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium); non-ionic block copolymers that form micelles such as CRL 1005 (these contain a linear chain of hydrophobic polyoxpropylene flanked by chains of polyoxyethylene; Vaxcel, Inc., Norcross, Ga.); and Syntex Adjuvant Formulation (SAF, an oil-in-water emulsion containing Tween 80 and a nonionic block copolymer; Syntex Chemicals, Inc., Boulder, Colo.).

The mucosal adjuvants useful according to the invention are adjuvants that are capable of inducing a mucosal immune response in a subject when administered to a mucosal surface in conjunction with complexes of the invention. Mucosal adjuvants include but are not limited to

CpG nucleic acids (e.g. PCT published patent application WO 99/61056), Bacterial toxins: e.g., Cholera toxin (CT), CT derivatives including but not limited to CT B subunit (CTB) (Wu et al., 1998, Tochikubo et al., 1998); CTD53 (Val to Asp) (Fontana et al., 1995); CTK97 (Val to Lys) (Fontana et al., 1995); CTK104 (Tyr to Lys) (Fontana et al., 1995); CTD53/K63 (Val to Asp, Ser to Lys) (Fontana et al., 1995); CTH54 (Arg to His) (Fontana et al., 1995); CTN107 (His to Asn) (Fontana et al., 1995); CTE114 (Ser to Glu) (Fontana et al., 1995); CTE112K (Glu to Lys) (Yamamoto et al., 1997a); CTS61F (Ser to Phe) (Yamamoto et al., 1997a, 1997b); CTS106 (Pro to Lys) (Douce et al., 1997, Fontana et al., 1995); and CTK63 (Ser to Lys) (Douce et al., 1997, Fontana et al., 1995), Zonula occludens toxin, zot, Escherichia coli heat-labile enterotoxin, Labile Toxin (LT), LT derivatives including but not limited to LT B subunit (LTB) (Verweij et al., 1998); LT7K (Arg to Lys) (Komase et al., 1998, Douce et al., 1995); LT61F (Ser to Phe) (Komase et al., 1998); LT112K (Glu to Lys) (Komase et al., 1998); LT118E (Gly to Glu) (Komase et al., 1998); LT146E (Arg to Glu) (Komase et al., 1998); LT192G (Arg to Gly) (Komase et al., 1998); LTK63 (Ser to Lys) (Marchetti et al., 1998, Douce et al., 1997, 1998, Di Tommaso et al., 1996); and LTR72 (Ala to Arg) (Giuliani et al., 1998), Pertussis toxin, PT. (Lycke et al., 1992, Spangler BD, 1992, Freytag and Clemments, 1999, Roberts et al., 1995, Wilson et al., 1995) including PT-9K/129G (Roberts et al., 1995, Cropley et al., 1995); Toxin derivatives (see below) (Holmgren et al., 1993, Verweij et al., 1998, Rappuoli et al., 1995, Freytag and Clements, 1999); Lipid A derivatives (e.g., monophosphoryl lipid A, MPL) (Sasaki et al., 1998, Vancott et al., 1998; Muramyl Dipeptide (MDP) derivatives (Fukushima et al., 1996, Ogawa et al., 1989, Michalek et al., 1983, Morisaki et al., 1983); bacterial outer membrane proteins (e.g., outer surface protein A (OspA) lipoprotein of Borrelia burgdorferi, outer membrane protine of Neisseria meningitidis)(Marinaro et al., 1999, Van de Verg et al., 1996); oil-in-water emulsions (e.g., MF59) (Barchfield et al., 1999, Verschoor et al., 1999, O'Hagan, 1998); aluminum salts (Isaka et al., 1998, 1999); and Saponins (e.g., QS21) Aquila Biopharmaceuticals, Inc., Worster, Me.) (Sasaki et al., 1998, MacNeal et al., 1998), ISCOMs, MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, Calif.); the Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720; AirLiquide, Paris, France); PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micell-forming agent; IDEC Pharmaceuticals Corporation, San Diego, Calif.); Syntext Adjuvant Formulation (SAF; Syntex Chemicals, Inc., Boulder, Colo.);

poly[di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA) and Leishmania elongation factor (Corixa Corporation, Seattle, Wash.).

### 4.5.1. TARGET CANCERS

In one embodiment, combination therapy encompasses, in addition to the administration of the complexes of the invention, the adjunctive uses of one or more modalities that aid in the prevention or treatment of cancer, which modalities include, but is not limited to chemotherapeutic agents, immunotherapeutics, anti-angiogenic agents, cytokines, hormones, antibodies, polynucleotides, radiation and photodynamic therapeutic agents. In specific embodiments, combination therapy can be used to prevent the recurrence of cancer, inhibit metastasis, or inhibit the growth and/or spread of cancer or metastasis.

Types of cancers that can be treated or prevented by the methods of the present invention include, but are not limited to human sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

In another embodiment, the patient having a cancer is immunosuppressed by reason of having undergone anti-cancer therapy (*e.g.*, chemotherapy radiation) prior to administration of

the HSP and/or α2M-peptide complexes or administration of the HSP- and/or α2M- sensitized APC.

There are many reasons why immunotherapy as provided by the present invention is desired for use in cancer patients. First, surgery with anesthesia may lead to immunosuppression. With appropriate immunotherapy in the preoperative period, this immunosuppression may be prevented or reversed. This could lead to fewer infectious complications and to accelerated wound healing. Second, tumor bulk is minimal following surgery and immunotherapy is most likely to be effective in this situation. A third reason is the possibility that tumor cells are shed into the circulation at surgery and effective immunotherapy applied at this time can eliminate these cells.

The preventive and therapeutic methods of the invention are directed at enhancing the immunocompetence of the cancer patient either before surgery, at or after surgery, and to induce tumor-specific immunity to cancer cells, with the objective being inhibition of cancer, and with the ultimate clinical objective being total cancer regression and eradication. The methods of the invention can also be used in individuals at enhanced risk of a particular type of cancer, *e.g.*, due to familial history or environmental risk factors.

In various embodiments, one or more anti-cancer agent, in addition to the complexes of the invention, is administered to treat a cancer patient. An anti-cancer agent refers to any molecule or compound that assists in the treatment of tumors or cancer. Examples of anti-cancer agents that may be used in the methods of the present invention include, but are not limited to: acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; ametantrone acetate; aminoglutethimide; amsacrine; anastrozole; anthramycin; asparaginase; asperlin; azacitidine; azetepa; azotomycin; batimastat; benzodepa; bicalutamide; bisantrene hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflornithine hydrochloride; elsamitrucin; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin

hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; interleukin II (including recombinant interleukin II, or rIL2), interferon alfa-2a; interferon alfa-2b; interferon alfa-n1 ; interferon alfa-n3; interferon beta-I a; interferon gamma-I b; iproplatin; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedepa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; piposulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprine; rogletimide; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin; spirogermanium hydrochloride; spiromustine; spiroplatin; streptonigrin; streptozocin; sulofenur; talisomycin; tecogalan sodium; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; toremifene citrate; trestolone acetate; triciribine phosphate; trimetrexate; trimetrexate glucuronate; triptorelin; tubulozole hydrochloride; uracil mustard; uredepa; vapreotide; verteporfin; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinglycinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; zorubicin hydrochloride.

Other anti-cancer drugs that can be used include, but are not limited to: 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene; adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic

acid; ara-CDP-DL-PTBA; arginine deaminase; asulacrine; atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzochlorins; benzoylstaurosporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; bFGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; bropirimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives; canarypox IL-2; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetrorelix; chlorlns; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentanthraquinones; cycloplatam; cypemycin; cytarabine ocfosfate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrodidemnin B; deslorelin; dexamethasone; dexifosfamide; dexrazoxane; dexverapamil; diaziquone; didemnin B; didox; diethylnorspermine; dihydro-5-azacytidine; dihydrotaxol, 9-; dioxamycin; diphenyl spiromustine; docetaxel; docosanol; dolasetron; doxifluridine; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin; epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole; etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride; flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorunicin hydrochloride; forfenimex; formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofosine; ilomastat; imidazoacridones; imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4-; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon; leuprolide+estrogen+progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; lovastatin;

loxoribine; lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maitansine; mannostatin A; marimastat; masoprocol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone; meterelin; methioninase; metoclopramide; MIF inhibitor; mifepristone; miltefosine; mirimostim; mismatched double stranded RNA; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim; monoclonal antibody, human chorionic gonadotrophin; monophosphoryl lipid A+myobacterium cell wall sk; mopidamol; multiple drug resistance gene inhibitor; multiple tumor suppressor 1-based therapy; mustard anti-cancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyldinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone+pentazocine; napavin; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; neutral endopeptidase; nilutamide; nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; O6-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel; paclitaxel analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate sodium; pentostatin; pentrozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride; pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum compounds; platinum-triamine complex; porfimer sodium; porfiromycin; prednisone; propyl bis-acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase inhibitors; purine nucleoside phosphorylase inhibitors; purpurins; pyrazoloacridine; pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron; ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rogletimide; rohitukine; romurtide; roquinimex; rubiginone B1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A; sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor 1; sense oligonucleotides; signal transduction inhibitors; signal transduction modulators; single chain antigen binding protein; sizofiran; sobuzoxane; sodium borocaptate; sodium phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D; spiromustine; splenopentin;

spongistatin 1; squalamine; stem cell inhibitor; stem-cell division inhibitors; stipiamide; stromelysin inhibitors; sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; synthetic glycosaminoglycans; tallimustine; tamoxifen methiodide; tauromustine; tazarotene; tecogalan sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; temozolomide; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene bichloride; topsentin; toremifene; totipotent stem cell factor; translation inhibitors; tretinoin; triacetyluridine; triciribine; trimetrexate; triptorelin; tropisetron; turosteride; tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vapreotide; variolin B; vector system, erythrocyte gene therapy; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; and zinostatin stimalamer.

An anti-cancer agent can be a chemotherapeutic agents which include but are not limited to, the following groups of compounds : cytotoxic antibiotics, antimetabolities, anti-mitotic agents, alkylating agents, platinum compounds, arsenic compounds, DNA topoisomerase inhibitors, taxanes, nucleoside analogues, plant alkaloids, and toxins; and synthetic derivatives thereof. Table 1 lists exemplary compounds of the groups:

TABLE 1

| | |
|---|---|
| Alkylating agents | |
| Nitrogen mustards: | Cyclophosphamide |
| | Ifosfamide |
| | Trofosfamide |
| | Chlorambucil |
| Nitrosoureas: | Carmustine (BCNU) |
| | Lomustine (CCNU) |
| Alkylsulphonates: | Busulfan |
| | Treosulfan |
| Triazenes: | Dacarbazine |
| Platinum containing compounds: | Cisplatin |
| | Carboplatin |
| | Aroplatin |
| | Oxaliplatin |
| Plant Alkaloids | |
| Vinca alkaloids: | Vincristine |

| | |
|---|---|
| | Vinblastine |
| | Vindesine |
| | Vinorelbine |
| Taxoids: | Paclitaxel |
| | Docetaxol |

DNA Topoisomerase Inhibitors

| | |
|---|---|
| Epipodophyllins: | Etoposide |
| | Teniposide |
| | Topotecan |
| | 9-aminocamptothecin |
| | Camptothecin |
| | Crisnatol |
| mitomycins: | Mitomycin C |

Anti-folates:

| | |
|---|---|
| DHFR inhibitors: | Methotrexate |
| | Trimetrexate |
| IMP dehydrogenase Inhibitors: | Mycophenolic acid |
| | Tiazofurin |
| | Ribavirin |
| | EICAR |
| Ribonuclotide reductase Inhibitors: | Hydroxyurea |
| | Deferoxamine |

Pyrimidine analogs:

| | |
|---|---|
| Uracil analogs: | 5-Fluorouracil |
| | Floxuridine |
| | Doxifluridine |
| | Ratitrexed |
| Cytosine analogs: | Cytarabine (ara C) |
| | Cytosine arabinoside |
| | Fludarabine |
| Purine analogs: | Mercaptopurine |
| | Thioguanine |

DNA Antimetabolites:

| |
|---|
| 3-HP |
| 2'-deoxy-5-fluorouridine |
| 5-HP |
| alpha-TGDR |
| aphidicolin glycinate |
| ara-C |
| 5-aza-2'-deoxycytidine |
| beta-TGDR |
| cyclocytidine |
| guanazole |
| inosine glycodialdehyde |

| | |
|---|---|
| | macebecin II |
| | pyrazoloimidazole |
| Antimitotic agents: | allocolchicine |
| | Halichondrin B |
| | colchicine |
| | colchicine derivative |
| | dolstatin 10 |
| | maytansine |
| | rhizoxin |
| | thiocolchicine |
| | trityl cysteine |
| Others: | |
| Isoprenylation inhibitors: | |
| Dopaminergic neurotoxins: | 1-methyl-4-phenylpyridinium ion |
| Cell cycle inhibitors: | Staurosporine |
| Actinomycins: | Actinomycin D |
| | Dactinomycin |
| Bleomycins: | Bleomycin A2 |
| | Bleomycin B2 |
| | Peplomycin |
| Anthracyclines: | Daunorubicin |
| | Doxorubicin (adriamycin) |
| | Idarubicin |
| | Epirubicin |
| | Pirarubicin |
| | Zorubicin |
| | Mitoxantrone |
| MDR inhibitors: | Verapamil |
| $Ca^{2+}$ATPase inhibitors: | Thapsigargin |

Compositions comprising one or more chemotherapeutic agents (*e.g.*, FLAG, CHOP) are also contemplated by the present invention. FLAG comprises fludarabine, cytosine arabinoside (Ara-C) and G-CSF. CHOP comprises cyclophosphamide, vincristine, doxorubicin, and prednisone. Each of the foregoing lists is illustrative, and is not intended to be limiting.

In one embodiment, breast cancer can be treated with a pharmaceutical composition comprising complexes of the invention in combination with 5-fluorouracil, cisplatin, docetaxel, doxorubicin, Herceptin®, gemcitabine, IL-2, paclitaxel, and/or VP-16 (etoposide).

In another embodiment, prostate cancer can be treated with a pharmaceutical composition comprising complexes of the invention in combination with paclitaxel, docetaxel, mitoxantrone, and/or an androgen receptor antagonist (*e.g.*, flutamide).

In another embodiment, leukemia can be treated with a pharmaceutical composition

comprising complexes of the invention in combination with fludarabine, cytosine arabinoside, gemtuzumab (MYLOTARG), daunorubicin, methotrexate, vincristine, 6-mercaptopurine, idarubicin, mitoxantrone, etoposide, asparaginase, prednisone and/or cyclophosphamide. As another example, myeloma can be treated with a pharmaceutical composition comprising complexes of the invention in combination with dexamethasone. Preferably, the leukemia is chronic myeloid leukemia (CML), the HSP complexes comprises hsp70-peptide complexes, and the therapeutic modality is imatinib mesylate or Gleevec™.

In another embodiment, melanoma can be treated with a pharmaceutical composition comprising complexes of the invention in combination with dacarbazine.

In another embodiment, colorectal cancer can be treated with a pharmaceutical composition comprising complexes of the invention in combination with irinotecan.

In another embodiment, lung cancer can be treated with a pharmaceutical composition comprising complexes of the invention in combination with paclitaxel, docetaxel, etoposide and/or cisplatin.

In another embodiment, non-Hodgkin's lymphoma can be treated with a pharmaceutical composition comprising complexes of the invention in combination with cyclophosphamide, CHOP, etoposide, bleomycin, mitoxantrone and/or cisplatin.

In another embodiment, gastric cancer can be treated with a pharmaceutical composition comprising complexes of the invention in combination with cisplatin.

In another embodiment, pancreatic cancer can be treated with a pharmaceutical composition comprising complexes of the invention in combination with gemcitabine.

According to the invention, the complexes of the invention can be administered prior to, subsequently, or concurrently with anti-cancer agent(s), for the prevention or treatment of cancer. Depending on the type of cancer, the subject's history and condition, and the anti-cancer agent(s) of choice, the use of the complexes of the invention can be coordinated with the dosage and timing of chemotherapy.

The use of the complexes of the invention can be added to a regimen of chemotherapy. In one embodiment, the chemotherapeutic agent is gemcitabine at a dose ranging from 100 to 1000 $mg/m^2$/cycle. In one embodiment, the chemotherapeutic agent is dacarbazine at a dose ranging from 200 to 4000 $mg/m^2$/cycle. In a preferred embodiment, the dose of dacarbazine ranges from 700 to 1000 $mg/m^2$/cycle. In another embodiment, the chemotherapeutic agent is fludarabine at a

dose ranging from 25 to 50 mg/m$^2$/cycle. In another embodiment, the chemotherapeutic agent is cytosine arabinoside (Ara-C) at a dose ranging from 200 to 2000 mg/m$^2$/cycle. In another embodiment, the chemotherapeutic agent is docetaxel at a dose ranging from 1.5 to 7.5 mg/kg/cycle. In another embodiment, the chemotherapeutic agent is paclitaxel at a dose ranging from 5 to 15 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is cisplatin at a dose ranging from 5 to 20 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is 5-fluorouracil at a dose ranging from 5 to 20 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is doxorubicin at a dose ranging from 2 to 8 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is epipodophyllotoxin at a dose ranging from 40 to 160 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is cyclophosphamide at a dose ranging from 50 to 200 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is irinotecan at a dose ranging from 50 to 75, 75 to 100, 100 to 125, or 125 to 150 mg/m$^2$/cycle. In yet another embodiment, the chemotherapeutic agent is vinblastine at a dose ranging from 3.7 to 5.4, 5.5 to 7.4, 7.5 to 11, or 11 to 18.5 mg/m$^2$/cycle. In yet another embodiment, the chemotherapeutic agent is vincristine at a dose ranging from 0.7 to 1.4, or 1.5 to 2 mg/m$^2$/cycle. In yet another embodiment, the chemotherapeutic agent is methotrexate at a dose ranging from 3.3 to 5, 5 to 10, 10 to 100, or 100 to 1000 mg/m$^2$/cycle.

In a preferred embodiment, the invention further encompasses the use of low doses of chemotherapeutic agents when administered as part of the combination therapy regimen. For example, initial treatment with the complexes of the invention increases the sensitivity of a tumor to subsequent challenge with a dose of chemotherapeutic agent, which dose is near or below the lower range of dosages when the chemotherapeutic agent is administered without complexes of the invention.

In one embodiment, complexes of the invention and a low dose (*e.g.*, 6 to 60 mg/m$^2$/day or less) of docetaxel are administered to a cancer patient. In another embodiment, complexes of the invention and a low dose (*e.g.*, 10 to 135 mg/m$^2$/day or less) of paclitaxel are administered to a cancer patient. In yet another embodiment, complexes of the invention and a low dose (*e.g.*, 2.5 to 25 mg/m$^2$/day or less) of fludarabine are administered to a cancer patient. In yet another embodiment, complexes of the invention and a low dose (*e.g.*, 0.5 to 1.5 g/m$^2$/day or less) of cytosine arabinoside (Ara-C) are administered to a cancer patient. In another embodiment, the chemotherapeutic agent is gemcitabine at a dose ranging from 10 to 100mg/m$^2$/cycle. In another

embodiment, the chemotherapeutic agent is cisplatin, *e.g.,* PLATINOL or PLATINOL-AQ (Bristol Myers), at a dose ranging from 5 to 10, 10 to 20, 20 to 40, or 40 to 75 mg/m$^2$/cycle. In yet another embodiment, a dose of cisplatin ranging from 7.5 to 75 mg/m$^2$/cycle is administered to a patient with ovarian cancer. In yet another embodiment, a dose of cisplatin ranging from 5 to 50 mg/m$^2$/cycle is administered to a patient with bladder cancer. In yet another embodiment, the chemotherapeutic agent is carboplatin, *e.g.,* PARAPLATIN (Bristol Myers), at a dose ranging from 2 to 4, 4 to 8, 8 to 16, 16 to 35, or 35 to 75 mg/m$^2$/cycle. In yet another embodiment, a dose of carboplatin ranging from 7.5 to 75 mg/m$^2$/cycle is administered to a patient with ovarian cancer. In another embodiment, a dose of carboplatin ranging from 5 to 50 mg/m$^2$/cycle is administered to a patient with bladder cancer. In yet another embodiment, a dose of carboplatin ranging from 2 to 20 mg/m$^2$/cycle is administered to a patient with testicular cancer. In yet another embodiment, the chemotherapeutic agent is docetaxel, *e.g.,* TAXOTERE (Rhone Poulenc Rorer) at a dose ranging from 6 to 10, 10 to 30, or 30 to 60 mg/m$^2$/cycle. In yet another embodiment, the chemotherapeutic agent is paclitaxel, *e.g.,* TAXOL (Bristol Myers Squibb), at a dose ranging from 10 to 20, 20 to 40, 40 to 70, or 70 to 135 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is 5-fluorouracil at a dose ranging from 0.5 to 5 mg/kg/cycle. In yet another embodiment, the chemotherapeutic agent is doxorubicin, *e.g.,* ADRIAMYCIN (Pharmacia & Upjohn), DOXIL (Alza), RUBEX (Bristol Myers Squibb), at a dose ranging from 2 to 4, 4 to 8, 8 to 15, 15 to 30, or 30 to 60 mg/kg/cycle.

In another embodiment, complexes of the invention is administered in combination with one or more immunotherapeutic agents, such as antibodies and vaccines. In a preferred embodiment, the antibodies have *in vivo* therapeutic and/or prophylactic uses against cancer. In some embodiments, the antibodies can be used for treatment and/or prevention of infectious disease. Examples of therapeutic and prophylactic antibodies include, but are not limited to, MDX-010 (Medarex, NJ) which is a humanized anti-CTLA-4 antibody currently in clinic for the treatment of prostate cancer; SYNAGIS® (MedImmune, MD) which is a humanized anti-respiratory syncytial virus (RSV) monoclonal antibody for the treatment of patients with RSV infection; HERCEPTIN® (Trastuzumab) (Genentech, CA) which is a humanized anti-HER2 monoclonal antibody for the treatment of patients with metastatic breast cancer. Other examples are a humanized anti-CD18 F(ab')$_2$ (Genentech); CDP860 which is a humanized anti-CD18 F(ab')$_2$ (Celltech, UK); PRO542 which is an anti-HIV gp120 antibody fused with CD4

(Progenics/Genzyme Transgenics); Ostavir which is a human anti Hepatitis B virus antibody (Protein Design Lab/Novartis); PROTOVIR™ which is a humanized anti-CMV IgG1 antibody (Protein Design Lab/Novartis); MAK-195 (SEGARD) which is a murine anti-TNF-α $F(ab')_2$ (Knoll Pharma/BASF); IC14 which is an anti-CD14 antibody (ICOS Pharm); a humanized anti-VEGF IgG1 antibody (Genentech); OVAREX™ which is a murine anti-CA 125 antibody (Altarex); PANOREX™ which is a murine anti-17-IA cell surface antigen IgG2a antibody (Glaxo Wellcome/Centocor); BEC2 which is a murine anti-idiotype (GD3 epitope) IgG antibody (ImClone System); IMC-C225 which is a chimeric anti-EGFR IgG antibody (ImClone System); VITAXIN™ which is a humanized anti-αVβ3 integrin antibody (Applied Molecular Evolution/MedImmune); Campath 1H/LDP-03 which is a humanized anti CD52 IgG1 antibody (Leukosite); Smart M195 which is a humanized anti-CD33 IgG antibody (Protein Design Lab/Kanebo); RITUXAN™ which is a chimeric anti-CD20 IgG1 antibody (IDEC Pharm/Genentech, Roche/Zettyaku); LYMPHOCIDE™ which is a humanized anti-CD22 IgG antibody (Immunomedics); Smart ID10 which is a humanized anti-HLA antibody (Protein Design Lab); ONCOLYM™ (Lym-1) is a radiolabelled murine anti-HLA DIAGNOSTIC REAGENT antibody (Techniclone); ABX-IL8 is a human anti-IL8 antibody (Abgenix); anti-CD11a is a humanized IgG1 antibody (Genentech/Xoma); ICM3 is a humanized anti-ICAM3 antibody (ICOS Pharm); IDEC-114 is a primatized anti-CD80 antibody (IDEC Pharm/Mitsubishi); ZEVALIN™ is a radiolabelled murine anti-CD20 antibody (IDEC/Schering AG); IDEC-131 is a humanized anti-CD40L antibody (IDEC/Eisai); IDEC-151 is a primatized anti-CD4 antibody (IDEC); IDEC-152 is a primatized anti-CD23 antibody (IDEC/Seikagaku); SMART anti-CD3 is a humanized anti-CD3 IgG (Protein Design Lab); 5G1.1 is a humanized anti-complement factor 5 (C5) antibody (Alexion Pharm); D2E7 is a humanized anti-TNF-α antibody (CAT/BASF); CDP870 is a humanized anti-TNF-α Fab fragment (Celltech); IDEC-151 is a primatized anti-CD4 IgG1 antibody (IDEC Pharm/SmithKline Beecham); MDX-CD4 is a human anti-CD4 IgG antibody (Medarex/Eisai/Genmab); CDP571 is a humanized anti-TNF-α IgG4 antibody (Celltech); LDP-02 is a humanized anti-α4β7 antibody (LeukoSite/Genentech); OrthoClone OKT4A is a humanized anti-CD4 IgG antibody (Ortho Biotech); ANTOVA™ is a humanized anti-CD40L IgG antibody (Biogen); ANTEGREN™ is a humanized anti-VLA-4 IgG antibody (Elan); MDX-33 is a human anti-CD64 (FcγR) antibody (Medarex/Centeon); SCH55700 is a humanized anti-IL-5 IgG4 antibody (Celltech/Schering); SB-240563 and SB-240683 are

humanized anti-IL-5 and IL-4 antibodies, respectively, (SmithKline Beecham); rhuMab-E25 is a humanized anti-IgE IgG1 antibody (Genentech/Norvartis/Tanox Biosystems); ABX-CBL is a murine anti CD-147 IgM antibody (Abgenix); BTI-322 is a rat anti-CD2 IgG antibody (Medimmune/Bio Transplant); Orthoclone/OKT3 is a murine anti-CD3 IgG2a antibody (ortho Biotech); SIMULECT™ is a chimeric anti-CD25 IgG1 antibody (Novartis Pharm); LDP-01 is a humanized anti-$\beta_2$-integrin IgG antibody (LeukoSite); Anti-LFA-1 is a murine anti CD18 F(ab')$_2$ (Pasteur-Merieux/Immunotech); CAT-152 is a human anti-TGF-$\beta_2$ antibody (Cambridge Ab Tech); and Corsevin M is a chimeric anti-Factor VII antibody (Centocor). The above-listed immunoreactive reagents, as well as any other immunoreactive reagents, may be administered according to any regimen known to those of skill in the art, including the regimens recommended by the suppliers of the immunoreactive reagents.

In another embodiment, complexes of the invention is administered in combination with one or more anti-angiogenic agents, which includes, but is not limited to, angiostatin, thalidomide, kringle 5, endostatin, Serpin (Serine Protease Inhibitor) anti-thrombin, 29 kDa N–terminal and a 40 kDa C-terminal proteolytic fragments of fibronectin, 16 kDa proteolytic fragment of prolactin, 7.8 kDa proteolytic fragment of platelet factor-4 , a 13-amino acid peptide corresponding to a fragment of platelet factor-4 (Maione et al., 1990, Cancer Res. 51:2077-2083), a 14-amino acid peptide corresponding to a fragment of collagen I (Tolma et al., 1993, J. Cell Biol. 122:497-511), a 19 amino acid peptide corresponding to a fragment of Thrombospondin I (Tolsma et al., 1993, J. Cell Biol. 122:497-511), a 20-amino acid peptide corresponding to a fragment of SPARC (Sage et al., 1995, J. Cell. Biochem. 57:1329-1334), or any fragments, family members, or variants thereof, including pharmaceutically acceptable salts thereof.

Other peptides that inhibit angiogenesis and correspond to fragments of laminin, fibronectin, procollagen, and EGF have also been described (*see, e.g.*, Cao, 1998, Prog Mol Subcell Biol. 20:161-176). Monoclonal antibodies and cyclic pentapeptides, which block certain integrins that bind RGD proteins (*i.e.*, possess the peptide motif Arg-Gly-Asp), have been demonstrated to have anti-vascularization activities (Brooks et al., 1994, Science 264:569-571; Hammes et al., 1996, Nature Medicine 2:529-533). Moreover, inhibition of the urokinase plasminogen activator receptor by receptor antagonists inhibits angiogenesis, tumor growth and metastasis (Min et al., 1996, Cancer Res. 56: 2428-33; Crowley et al., 1993, Proc Natl Acad Sci. 90:5021-25). Use of such anti-angiogenic agents in combination with the complexes is also

contemplated by the present invention.

In yet another embodiment, complexes of the invention is used in association with a hormonal treatment. Hormonal therapeutic treatments comprise hormonal agonists, hormonal antagonists (*e.g.*, flutamide, bicalutamide, tamoxifen, raloxifene, leuprolide acetate (LUPRON), LH-RH antagonists), inhibitors of hormone biosynthesis and processing, and steroids (*e.g.*, dexamethasone, retinoids, deltoids, betamethasone, cortisol, cortisone, prednisone, dehydrotestosterone, glucocorticoids, mineralocorticoids, estrogen, testosterone, progestins), vitamin A derivatives (e.g., all-trans retinoic acid (ATRA)); vitamin D3 analogs; antigestagens (*e.g.*, mifepristone, onapristone), and antiandrogens (*e.g.*, cyproterone acetate).

In yet another embodiment, complexes of the invention are used in association with a gene therapy program in the treatment of cancer. In one embodiment, gene therapy with recombinant cells secreting interleukin-2 is administered in combination with complexes of the invention to prevent or treat cancer, particularly breast cancer (*See, e.g.*, Deshmukh et al., 2001, J Neurosurg. 94:287-92). In other embodiments, gene therapy is conducted with the use of polynucleotide compounds, such as but not limited to antisense polynucleotides, ribozymes, RNA interference molecules, triple helix polynucleotides and the like, where the nucleotide sequence of such compounds are related to the nucleotide sequences of DNA and/or RNA of genes that are linked to the initiation, progression, and/or pathology of a tumor or cancer. For example, many are oncogenes, growth factor genes, growth factor receptor genes, cell cycle genes, DNA repair genes, and are well known in the art.

In another embodiment, complexes of the invention is administered in conjunction with a regimen of radiation therapy. For radiation treatment, the radiation can be gamma rays or X-rays. The methods encompass treatment of cancer comprising radiation therapy, such as external-beam radiation therapy, interstitial implantation of radioisotopes (I-125, palladium, iridium), radioisotopes such as strontium-89, thoracic radiation therapy, intraperitoneal P-32 radiation therapy, and/or total abdominal and pelvic radiation therapy. For a general overview of radiation therapy, see Hellman, Chapter 16: Principles of Cancer Management: Radiation Therapy, 6th edition, 2001, DeVita *et al.*, eds., J.B. Lippencott Company, Philadelphia. In preferred embodiments, the radiation treatment is administered as external beam radiation or teletherapy wherein the radiation is directed from a remote source. In various preferred embodiments, the radiation treatment is administered as internal therapy or brachytherapy wherein a radiaoactive

source is placed inside the body close to cancer cells or a tumor mass. Also encompassed is the combined use of complexes of the invention with photodynamic therapy comprising the administration of photosensitizers, such as hematoporphyrin and its derivatives, Vertoporfin (BPD-MA), phthalocyanine, photosensitizer Pc4, demethoxy-hypocrellin A; and 2BA-2-DMHA.

In various embodiments, complexes of the invention is administered, in combination with at least one chemotherapeutic agent, for a short treatment cycle to a cancer patient to treat cancer. The duration of treatment with the chemotherapeutic agent may vary according to the particular cancer therapeutic agent used. The invention also contemplates discontinuous administration or daily doses divided into several partial administrations. An appropriate treatment time for a particular cancer therapeutic agent will be appreciated by the skilled artisan, and the invention contemplates the continued assessment of optimal treatment schedules for each cancer therapeutic agent. The present invention contemplates at least one cycle, preferably more than one cycle during which a single therapeutic or sequence of therapeutics is administered. An appropriate period of time for one cycle will be appreciated by the skilled artisan, as will the total number of cycles, and the interval between cycles.

In another embodiment, complexes of the invention are used in combination with compounds that ameliorate the symptoms of the cancer (such as but not limited to pain) and the side effects produced by the complexes of the invention (such as but not limited to flu-like symptoms, fever, etc). Accordingly, many compounds known to reduce pain, flu-like symptoms, and fever can be used in combination or in admixture with complexes of the invention. Such compounds include analgesics (e.g, acetaminophen), decongestants (e.g., pseudoephedrine), antihistamines (e.g., chlorpheniramine maleate), and cough suppressants (e.g., dextromethorphan).

### 4.5.2. TARGET INFECTIOUS DISEASES

Infectious diseases that can be treated or prevented by the methods of the present invention are caused by infectious agents including, but not limited to, viruses, bacteria, fungi protozoa, helminths, and parasites. The invention is not limited to treating or preventing infectious diseases caused by intracellular pathogens. Many medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, Medical Microbiology, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

Combination therapy encompasses in addition to the administration of complexes of the invention, the uses of one or more modalities that aid in the prevention or treatment of infectious diseases, which modalities include, but is not limited to antibiotics, antivirals, antiprotozoal compounds, antifungal compounds, and antihelminthics. Other treatment modalities that can be used to treat or prevent infectious diseases include immunotherapeutics, polynucleotides, antibodies, cytokines, and hormones as described above.

Infectious virus of both human and non-human vertebrates, include retroviruses, RNA viruses and DNA viruses. Examples of virus that have been found in humans include but are not limited to: Retroviridae (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bungaviridae (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g. reoviruses, orbiviurses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvovirida (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Retroviruses that are contemplated include both simple retroviruses and complex retroviruses. The simple retroviruses include the subgroups of B-type retroviruses, C-type retroviruses and D-type retroviruses. An example of a B-type retrovirus is mouse mammary tumor virus (MMTV). The C-type retroviruses include subgroups C-type group A (including Rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian myeloblastosis virus (AMV))

and C-type group B (including murine leukemia virus (MLV), feline leukemia virus (FeLV), murine sarcoma virus (MSV), gibbon ape leukemia virus (GALV), spleen necrosis virus (SNV), reticuloendotheliosis virus (RV) and simian sarcoma virus (SSV)). The D-type retroviruses include Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1). The complex retroviruses include the subgroups of lentiviruses, T-cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, SIV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T-cell leukemia viruses include HTLV-1, HTLV-II, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Examples of RNA viruses that are antigens in vertebrate animals include, but are not limited to, the following: members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus, Eugenangee virus, Kemerovo virus, African horse sickness virus, and Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, murine rotavirus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picornaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A virus, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus muris, Bovine enteroviruses, Porcine enteroviruses, the genus Cardiovirus (Encephalomyocarditis virus (EMC), Mengovirus), the genus Rhinovirus (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Apthovirus (Foot and Mouth disease (FMDV); the family Calciviridae, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavirius (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyvirus

(Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavirius (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus; Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus,, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyvirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); the family Rhabdoviridae, including the genus Vesiculovirus (VSV), Chandipura virus, Flanders-Hart Park virus), the genus Lyssavirus (Rabies virus), fish Rhabdoviruses, and two probable Rhabdoviruses (Marburg virus

and Ebola virus); the family Arenaviridae, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family Coronoaviridae, including Infectious Bronchitis Virus (IBV), Mouse Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that are antigens in vertebrate animals include, but are not limited to: the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia), the genus Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowlpox, other avian poxvirus), the genus Capripoxvirus (sheeppox, goatpox), the genus Suipoxvirus (Swinepox), the genus Parapoxvirus (contagious postular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesviruses (Human cytomegalovirus and cytomegaloviruses of swine, monkeys and rodents); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus ateles, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A,B,C,D,E and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivatable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotrophic papilloma virus); the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc). Finally, DNA viruses may include viruses which do not fit into the above families such as Kuru and Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents.

Many examples of antiviral compounds that can be used in combination with the complexes of the invention are known in the art and include but are not limited to: rifampicin, nucleoside reverse transcriptase inhibitors (e.g., AZT, ddI, ddC, 3TC, d4T), non-nucleoside reverse transcriptase inhibitors (e.g., Efavirenz, Nevirapine), protease inhibitors (e.g., aprenavir, indinavir, ritonavir, and saquinavir), idoxuridine, cidofovir, acyclovir, ganciclovir, zanamivir, amantadine, and Palivizumab. Other examples of anti-viral agents include but are not limited to Acemannan; Acyclovir; Acyclovir Sodium; Adefovir; Alovudine; Alvircept Sudotox; Amantadine Hydrochloride; Aranotin; Arildone; Atevirdine Mesylate; Avridine; Cidofovir; Cipamfylline; Cytarabine Hydrochloride; Delavirdine Mesylate; Desciclovir; Didanosine; Disoxaril; Edoxudine; Enviradene; Enviroxime; Famciclovir; Famotine Hydrochloride; Fiacitabine; Fialuridine; Fosarilate; Foscamet Sodium; Fosfonet Sodium; Ganciclovir; Ganciclovir Sodium; Idoxuridine; Kethoxal; Lamivudine; Lobucavir; Memotine Hydrochloride; Methisazone; Nevirapine; Penciclovir; Pirodavir; Ribavirin; Rimantadine Hydrochloride; Saquinavir Mesylate; Somantadine Hydrochloride; Sorivudine; Statolon; Stavudine; Tilorone Hydrochloride; Trifluridine; Valacyclovir Hydrochloride; Vidarabine; Vidarabine Phosphate; Vidarabine Sodium Phosphate; Viroxime; Zalcitabine; Zidovudine; Zinviroxime.

Bacterial infections or diseases that can be treated or prevented by the methods of the present invention are caused by bacteria including, but not limited to, bacteria that have an intracellular stage in its life cycle, such as mycobacteria (e.g., *Mycobacteria tuberculosis*, *M. bovis*, *M. avium*, *M. leprae*, or *M. africanum*), rickettsia, mycoplasma, chlamydia, and legionella. Other examples of bacterial infections contemplated include but are not limited to infections caused by Gram positive bacillus (e.g., *Listeria*, *Bacillus* such as *Bacillus anthracis*, *Erysipelothrix* species), Gram negative bacillus (e.g., *Bartonella*, *Brucella*, *Campylobacter*, *Enterobacter*, *Escherichia*, *Francisella*, *Hemophilus*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio*, and *Yersinia* species), spirochete bacteria (e.g., *Borrelia* species including *Borrelia burgdorferi* that causes Lyme disease), anaerobic bacteria (e.g., *Actinomyces* and *Clostridium* species), Gram positive and negative coccal bacteria, *Enterococcus* species, *Streptococcus* species, *Pneumococcus* species, *Staphylococcus* species, *Neisseria* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pyloris*, *Borelia burgdorferi*, *Legionella pneumophilia*, *Mycobacteria tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*,

*Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus viridans, Streptococcus faecalis, Streptococcus bovis, Streptococcus pneumoniae, Haemophilus influenzae, Bacillus antracis, corynebacterium diphtheriae, Erysipelothrix rhusiopathiae, Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida, Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira, Rickettsia,* and *Actinomyces israelli.*

Antibacterial agents or antibiotics that can be used in combination with the complexes of the invention include but are not limited to: aminoglycoside antibiotics (*e.g.*, apramycin, arbekacin, bambermycins, butirosin, dibekacin, neomycin, neomycin, undecylenate, netilmicin, paromomycin, ribostamycin, sisomicin, and spectinomycin), amphenicol antibiotics (*e.g.*, azidamfenicol, chloramphenicol, florfenicol, and thiamphenicol), ansamycin antibiotics (*e.g.*, rifamide and rifampin), carbacephems (*e.g.*, loracarbef), carbapenems (*e.g.*, biapenem and imipenem), cephalosporins (*e.g.*, cefaclor, cefadroxil, cefamandole, cefatrizine, cefazedone, cefozopran, cefpimizole, cefpiramide, and cefpirome), cephamycins (*e.g.*, cefbuperazone, cefmetazole, and cefminox), monobactams (*e.g.*, aztreonam, carumonam, and tigemonam), oxacephems (*e.g.*, flomoxef, and moxalactam), penicillins (*e.g.*, amdinocillin, amdinocillin pivoxil, amoxicillin, bacampicillin, benzylpenicillinic acid, benzylpenicillin sodium, epicillin, fenbenicillin, floxacillin, penamccillin, penethamate hydriodide, penicillin o-benethamine, penicillin 0, penicillin V, penicillin V benzathine, penicillin V hydrabamine, penimepicycline, and phencihicillin potassium), lincosamides (*e.g.*, clindamycin, and lincomycin), macrolides (*e.g.*, azithromycin, carbomycin, clarithomycin, dirithromycin, erythromycin, and erythromycin acistrate), amphomycin, bacitracin, capreomycin, colistin, enduracidin, enviomycin, tetracyclines (*e.g.*, apicycline, chlortetracycline, clomocycline, and demeclocycline), 2,4-diaminopyrimidines (*e.g.*, brodimoprim), nitrofurans (*e.g.*, furaltadone, and furazolium chloride), quinolones and analogs thereof (e.g., cinoxacin, ciprofloxacin, clinafloxacin, flumequine, and grepagloxacin), sulfonamides (*e.g.*, acetyl sulfamethoxypyrazine, benzylsulfamide, noprylsulfamide, phthalylsulfacetamide, sulfachrysoidine, and sulfacytine), sulfones (*e.g.*, diathymosulfone, glucosulfone sodium, and solasulfone), cycloserine, mupirocin and tuberin.

Additional examples of antibacterial agents include but are not limited to Acedapsone;

Acetosulfone Sodium; Alamecin; Alexidine; Amdinocillin; Amdinocillin Pivoxil; Amicycline; Amifloxacin; Amifloxacin Mesylate; Amikacin; Amikacin Sulfate; Aminosalicylic acid; Aminosalicylate sodium; Amoxicillin; Amphomycin; Ampicillin; Ampicillin Sodium; Apalcillin Sodium; Apramycin; Aspartocin; Astromicin Sulfate; Avilamycin; Avoparcin; Azithromycin; Azlocillin; Azlocillin Sodium; Bacampicillin Hydrochloride; Bacitracin; Bacitracin Methylene Disalicylate; Bacitracin Zinc; Bambermycins; Benzoylpas Calcium; Berythromycin; Betamicin Sulfate; Biapenem; Biniramycin; Biphenamine Hydrochloride; Bispyrithione Magsulfex; Butikacin; Butirosin Sulfate; Capreomycin Sulfate; Carbadox; Carbenicillin Disodium; Carbenicillin Indanyl Sodium; Carbenicillin Phenyl Sodium; Carbenicillin Potassium; Carumonam Sodium; Cefaclor; Cefadroxil; Cefamandole; Cefamandole Nafate; Cefamandole Sodium; Cefaparole; Cefatrizine; Cefazaflur Sodium; Cefazolin; Cefazolin Sodium; Cefbuperazone; Cefdinir; Cefepime; Cefepime Hydrochloride; Cefetecol; Cefixime; Cefmnenoxime Hydrochloride; Cefmetazole; Cefmetazole Sodium; Cefonicid Monosodium; Cefonicid Sodium; Cefoperazone Sodium; Ceforanide; Cefotaxime Sodium; Cefotetan; Cefotetan Disodium; Cefotiam Hydrochloride; Cefoxitin; Cefoxitin Sodium; Cefpimizole; Cefpimizole Sodium; Cefpiramide; Cefpiramide Sodium; Cefpirome Sulfate; Cefpodoxime Proxetil; Cefprozil; Cefroxadine; Cefsulodin Sodium; Ceftazidime; Ceftibuten; Ceftizoxime Sodium; Ceftriaxone Sodium; Cefuroxime; Cefuroxime Axetil; Cefuroxime Pivoxetil; Cefuroxime Sodium; Cephacetrile Sodium; Cephalexin; Cephalexin Hydrochloride; Cephaloglycin; Cephaloridine; Cephalothin Sodium; Cephapirin Sodium; Cephradine; Cetocycline Hydrochloride; Cetophenicol; Chloramphenicol; Chloramphenicol Palmitate; Chloramphenicol Pantothenate Complex; Chloramphenicol Sodium Succinate; Chlorhexidine Phosphanilate; Chloroxylenol; Chlortetracycline Bisulfate; Chlortetracycline Hydrochloride; Cinoxacin; Ciprofloxacin; Ciprofloxacin Hydrochloride; Cirolemycin; Clarithromycin; Clinafloxacin Hydrochloride; Clindamycin; Clindamycin Hydrochloride; Clindamycin Palmitate Hydrochloride; Clindamycin Phosphate; Clofazimine; Cloxacillin Benzathine; Cloxacillin Sodium; Cloxyquin; Colistimethate Sodium; Colistin Sulfate; Coumermycin; Coumermycin Sodium; Cyclacillin; Cycloserine; Dalfopristin; Dapsone; Daptomycin; Demeclocycline; Demeclocycline Hydrochloride; Demecycline; Denofungin; Diaveridine; Dicloxacillin; Dicloxacillin Sodium; Dihydrostreptomycin Sulfate; Dipyrithione; Dirithromycin; Doxycycline; Doxycycline Calcium; Doxycycline Fosfatex; Doxycycline Hyclate; Droxacin Sodium; Enoxacin;

Epicillin; Epitetracycline Hydrochloride; Erythromycin; Erythromycin Acistrate; Erythromycin Estolate; Erythromycin Ethylsuccinate; Erythromycin Gluceptate; Erythromycin Lactobionate; Erythromycin Propionate; Erythromycin Stearate; Ethambutol Hydrochloride; Ethionamide; Fleroxacin; Floxacillin; Fludalanine; Flumequine; Fosfomycin; Fosfomycin Tromethamine; Fumoxicillin; Furazolium Chloride; Furazolium Tartrate; Fusidate Sodium; Fusidic Acid; Gentamicin Sulfate; Gloximonam; Gramicidin; Haloprogin; Hetacillin; Hetacillin Potassium; Hexedine; Ibafloxacin; Imipenem; Isoconazole; Isepamicin; Isoniazid; Josamycin; Kanamycin Sulfate; Kitasamycin; Levofuraltadone; Levopropylcillin Potassium; Lexithromycin; Lincomycin; Lincomycin Hydrochloride; Lomefloxacin; Lomefloxacin Hydrochloride; Lomefloxacin Mesylate; Loracarbef; Mafenide; Meclocycline; Meclocycline Sulfosalicylate; Megalomicin Potassium Phosphate; Mequidox; Meropenem; Methacycline; Methacycline Hydrochloride; Methenamine; Methenamine Hippurate; Methenamine Mandelate; Methicillin Sodium; Metioprim; Metronidazole Hydrochloride; Metronidazole Phosphate; Mezlocillin; Mezlocillin Sodium; Minocycline; Minocycline Hydrochloride; Mirincamycin Hydrochloride; Monensin; Monensin Sodium; Nafcillin Sodium; Nalidixate Sodium; Nalidixic Acid; Natamycin; Nebramycin; Neomycin Palmitate; Neomycin Sulfate; Neomycin Undecylenate; Netilmicin Sulfate; Neutramycin; Nifuradene; Nifuraldezone; Nifuratel; Nifuratrone; Nifurdazil; Nifurimide; Nifurpirinol; Nifurquinazol; Nifurthiazole; Nitrocycline; Nitrofurantoin; Nitromide; Norfloxacin; Novobiocin Sodium; Ofloxacin; Ormetoprim; Oxacillin Sodium; Oximonam; Oximonam Sodium; Oxolinic Acid; Oxytetracycline; Oxytetracycline Calcium; Oxytetracycline Hydrochloride; Paldimycin; Parachlorophenol; Paulomycin; Pefloxacin; Pefloxacin Mesylate; Penamecillin; Penicillin G Benzathine; Penicillin G Potassium; Penicillin G Procaine; Penicillin G Sodium; Penicillin V; Penicillin V Benzathine; Penicillin V Hydrabamine; Penicillin V Potassium; Pentizidone Sodium; Phenyl Aminosalicylate; Piperacillin Sodium; Pirbenicillin Sodium; Piridicillin Sodium; Pirlimycin Hydrochloride; Pivampicillin Hydrochloride; Pivampicillin Pamoate; Pivampicillin Probenate; Polymyxin B Sulfate; Porfiromycin; Propikacin; Pyrazinamide; Pyrithione Zinc; Quindecamine Acetate; Quinupristin; Racephenicol; Ramoplanin; Ranimycin; Relomycin; Repromicin; Rifabutin; Rifametane; Rifamexil; Rifamide; Rifampin; Rifapentine; Rifaximin; Rolitetracycline; Rolitetracycline Nitrate; Rosaramicin; Rosaramicin Butyrate; Rosaramicin Propionate; Rosaramicin Sodium Phosphate; Rosaramicin Stearate; Rosoxacin; Roxarsone; Roxithromycin; Sancycline; Sanfetrinem Sodium; Sarmoxicillin;

Sarpicillin; Scopafingin; Sisomicin; Sisomicin Sulfate; Sparfloxacin; Spectinomycin Hydrochloride; Spiramycin; Stallimycin Hydrochloride; Steffimycin; Streptomycin Sulfate; Streptonicozid; Sulfabenz; Sulfabenzamide; Sulfacetamide; Sulfacetamide Sodium; Sulfacytine; Sulfadiazine; Sulfadiazine Sodium; Sulfadoxine; Sulfalene; Sulfamerazine; Sulfameter; Sulfamethazine; Sulfamethizole; Sulfamethoxazole; Sulfamonomethoxine; Sulfamoxole; Sulfanilate Zinc; Sulfanitran; Sulfasalazine; Sulfasomizole; Sulfathiazole; Sulfazamet; Sulfisoxazole; Sulfisoxazole Acetyl; Sulfisoxazole Diolamine; Sulfomyxin; Sulopenem; Sultamicillin; Suncillin Sodium; Talampicillin Hydrochloride; Teicoplanin; Temafloxacin Hydrochloride; Temocillin; Tetracycline; Tetracycline Hydrochloride; Tetracycline Phosphate Complex; Tetroxoprim; Thiamphenicol; Thiphencillin Potassium; Ticarcillin Cresyl Sodium; Ticarcillin Disodium; Ticarcillin Monosodium; Ticlatone; Tiodonium Chloride; Tobramycin; Tobramycin Sulfate; Tosufloxacin; Trimethoprim; Trimethoprim Sulfate; Trisulfapyrimidines; Troleandomycin; Trospectomycin Sulfate; Tyrothricin; Vancomycin; Vancomycin Hydrochloride; Virginiamycin; Zorbamycin.

Fungal diseases that can be treated or prevented by the methods of the present invention include but not limited to aspergilliosis, crytococcosis, sporotrichosis, coccidioidomycosis, paracoccidioidomycosis, histoplasmosis, blastomycosis, zygomycosis, and candidiasis.

Antifungal compounds that can be used in combination with the complexes of the invention include but are not limited to: polyenes (*e.g.,* amphotericin b, candicidin, mepartricin, natamycin, and nystatin), allylamines (*e.g.,* butenafine, and naftifine), imidazoles (*e.g.,* bifonazole, butoconazole, chlordantoin, flutrimazole, isoconazole, ketoconazole, and lanoconazole), thiocarbamates (*e.g.,* tolciclate, tolindate, and tolnaftate), triazoles (*e.g.,* fluconazole, itraconazole, saperconazole, and terconazole), bromosalicylchloranilide, buclosamide, calcium propionate, chlorphenesin, ciclopirox, azaserine, griseofulvin, oligomycins, neomycin undecylenate, pyrrolnitrin, siccanin, tubercidin, and viridin. Additional examples of antifungal compounds include but are not limited to Acrisorcin; Ambruticin; Amphotericin B; Azaconazole; Azaserine; Basifungin; Bifonazole; Biphenamine Hydrochloride; Bispyrithione Magsulfex; Butoconazole Nitrate; Calcium Undecylenate; Candicidin; Carbol-Fuchsin; Chlordantoin; Ciclopirox; Ciclopirox Olamine; Cilofungin; Cisconazole; Clotrimazole; Cuprimyxin; Denofungin; Dipyrithione; Doconazole; Econazole; Econazole Nitrate; Enilconazole; Ethonam Nitrate; Fenticonazole Nitrate; Filipin; Fluconazole; Flucytosine;

Fungimycin; Griseofulvin; Hamycin; Isoconazole; Itraconazole; Kalafungin; Ketoconazole; Lomofingin; Lydimycin; Mepartricin; Miconazole; Miconazole Nitrate; Monensin; Monensin Sodium; Naftifine Hydrochloride; Neomycin Undecylenate; Nifuratel; Nifurmerone; Nitralamine Hydrochloride; Nystatin; Octanoic Acid; Orconazole Nitrate; Oxiconazole Nitrate; Oxifungin Hydrochloride; Parconazole Hydrochloride; Partricin; Potassium Iodide; Proclonol; Pyrithione Zinc; Pyrrolnitrin; Rutamycin; Sanguinarium Chloride; Saperconazole; Scopafungin; Selenium Sulfide; Sinefungin; Sulconazole Nitrate; Terbinafine; Terconazole; Thiram; Ticlatone; Tioconazole; Tolciclate; Tolindate; Tolnaftate; Triacetin; Triafuigin; Undecylenic Acid; Viridoflilvin; Zinc Undecylenate; and Zinoconazole Hydrochloride.

Parasitic diseases that can be treated or prevented by the methods of the present invention including, but not limited to, amebiasis, malaria, leishmania, coccidia, giardiasis, cryptosporidiosis, toxoplasmosis, and trypanosomiasis. Also encompassed are infections by various worms, such as but not limited to ascariasis, ancylostomiasis, trichuriasis, strongyloidiasis, toxoccariasis, trichinosis, onchocerciasis. filaria, and dirofilariasis. Also encompassed are infections by various flukes, such as but not limited to schistosomiasis, paragonimiasis, and clonorchiasis. Parasites that cause these diseases can be classified based on whether they are intracellular or extracellular. An "intracellular parasite" as used herein is a parasite whose entire life cycle is intracellular. Examples of human intracellular parasites include *Leishmania* spp., *Plasmodium* spp., *Trypanosoma cruzi, Toxoplasma gondii, Babesia* spp., and *Trichinella spiralis*. An "extracellular parasite" as used herein is a parasite whose entire life cycle is extracellular. Extracellular parasites capable of infecting humans include *Entamoeba histolytica, Giardia lamblia, Enterocytozoon bieneusi, Naegleria* and *Acanthamoeba* as well as most helminths. Yet another class of parasites is defined as being mainly extracellular but with an obligate intracellular existence at a critical stage in their life cycles. Such parasites are referred to herein as "obligate intracellular parasites". These parasites may exist most of their lives or only a small portion of their lives in an extracellular environment, but they all have at least one obligate intracellular stage in their life cycles. This latter category of parasites includes *Trypanosoma rhodesiense* and *Trypanosoma gambiense, Isospora* spp., *Cryptosporidium* spp, *Eimeria* spp., *Neospora* spp., *Sarcocystis* spp., and *Schistosoma* spp.

. Many examples of antiprotozoal compounds that can be used in combination with the complexes of the invention to treat parasitic diseases are known in the art and include but are not

limited to: quinines, chloroquine, mefloquine, proguanil, pyrimethamine, metronidazole, diloxanide furoate, tinidazole, amphotericin, sodium stibogluconate, trimoxazole, and pentamidine isetionate. Many examples of antiparasite drugs that can be used in combination with the complexes of the invention to treat parasitic diseases are known in the art and include but are not limited to: mebendazole, levamisole, niclosamide, praziquantel, albendazole, ivermectin, diethylcarbamazine, and thiabendazole. Further examples of anti-parasitic compounds include but are not limited to Acedapsone; Amodiaquine Hydrochloride; Amquinate; Arteflene; Chloroquine; Chloroquine Hydrochloride; Chloroquine Phosphate; Cycloguanil Pamoate; Enpiroline Phosphate; Halofantrine Hydrochloride; Hydroxychloroquine Sulfate; Mefloquine Hydrochloride; Menoctone; Mirincamycin Hydrochloride; Primaquine Phosphate; Pyrimethamine; Quinine Sulfate; and Tebuquine.

In a less preferred embodiment, the complexes of the invention can be used in combination with a non-HSP and non-α2M-based vaccine composition. Examples of such vaccines for humans are described in The Jordan Report 2000, Accelerated Development of Vaccines, National Institute of Health, which is incorporated herein by reference in its entirety. Many vaccines for the treatment of non-human vertebrates are disclosed in Bennett, K. Compendium of Veterinary Products, 3rd ed. North American Compendiums, Inc., 1995, which is incorporated herein by reference in its entirety.

### 4.5.3. AUTOLOGOUS EMBODIMENT

The specific immunogenicity of HSPs and α2M derives not from HSPs or α2M per se, but from the antigenic proteins and/or peptides bound to them. In a preferred embodiment of the invention, the complexes in the compositions of the inventions for use as cancer vaccines are autologous complexes, thereby circumventing two of the most intractable hurdles to cancer immunotherapy. First is the possibility that human cancers, like cancers of experimental animals, are antigenically distinct. To circumvent this hurdle, in a preferred embodiment of the present invention, the HSPs and/or α2M are complexed to antigenic proteins and peptides, and the complexes are used to treat the cancers in the same subject from which the proteins or peptides are derived. Second, most current approaches to cancer immunotherapy focus on determining the CTL-recognized epitopes of cancer cell lines. This approach requires the availability of cell lines and CTLs against cancers. These reagents are unavailable for an overwhelming proportion of

human cancers. In an embodiment of the present invention directed to the use of autologous antigenic proteins and/or peptides, cancer immunotherapy does not depend on the availability of cell lines or CTLs nor does it require definition of the antigenic epitopes of cancer cells. These advantages make complexes of HSPs and/or α2M bound to autologous antigenic proteins and/or peptides attractive immunogens against cancer.

In other embodiments, the antigenic peptides in the therapeutic or prophylactic complexes can be prepared from cancerous tissue of the same type of cancer from a subject allogeneic to the subject to whom the complexes are administered.

## 4.6. ADOPTIVE IMMUNOTHERAPY

Adoptive immunotherapy refers to a therapeutic approach for treating cancer or infectious diseases in which immune cells are administered to a host with the aim that the cells mediate either directly or indirectly specific immunity to tumor cells and/or antigenic components or regression of the tumor or treatment of infectious diseases, as the case may be. (See e.g., U.S. Patent No. 5,985,270, issued November 16, 1999, which is incorporated by reference herein in its entirety).

In one embodiment, antigen presenting cells (APC) for use in adoptive immunotherapy are sensitized with HSPs and/or α2M complexed with antigenic proteins and peptides prepared in accordance with the methods described herein. The complexes can be produced by complexing heat shock protein or alpha-2-macroglobulin to antigenic proteins that are derived from at least 50% of the different proteins or at least 100 different proteins present in antigenic cells or viral particles that express an antigenic determinant of an agent that causes the infectious disease. The complexes can also be produced by (a) subjecting a protein preparation derived from cells of said type of cancer to either digestion with a protease or contact with ATP, guanidium hydrochloride, and/or acid, to generate a population of antigenic peptides, and (b) complexing the population of antigenic peptides to heat shock protein or alpha-2-macroglobulin.

In another embodiment, therapy by administration of in vitro complexed antigenic peptides and HSPs and/or α2M prepared by the methods of the invention may be combined with adoptive immunotherapy using APC sensitized by HSP- and/or α2M-antigenic peptide complexes prepared by any method known in the art (see e.g., U.S. Patent No. 5,985,270) in which the antigenic peptides display the desired antigenicity (e.g., of the type of cancer or

pathogen). The sensitized APC can be administered alone, in combination with the in vitro complexed proteins/peptides and HSPs and/or α2M, or before or after administration of the complexed proteins/peptides and HSPs and/or α2M. In particular, the use of sensitized APC to prevent and treat cancer can further comprise administering to the subject an amount, effective for said treatment or prevention, of complexes comprising heat shock protein and/or alpha-2-macroglobulin, complexed to antigenic proteins/peptides, wherein said complexes were produced as described above. Similarly, the use of sensitized APC in treating or preventing a type of infectious disease, can further comprise administering to the subject an amount, effective for said treatment or prevention, of complexes comprising heat shock protein and/or alpha-2-macroglobulin, complexed to antigenic proteins/peptides.

Furthermore, the mode of administration of the in vitro complexed antigenic proteins/peptides and HSPs and/or α2M can be varied, including but not limited to, *e.g.*, subcutaneously, intravenously or intramuscularly, although intradermally is preferred. In another specific embodiment, adoptive immunotherapy by administration of the antigen presenting cells sensitized with complexes made according to the present invention can be combined with therapy by administration by HSP- and/or α2M-antigenic molecule (e.g., peptide) complexes prepared by any method known in the art (see e.g., U.S. Patent No. 5,750,119, 5,837,251, 5,961,979, 5,935,576, PCT publications WO 94/14976 or WO 99/50303) in which the antigenic molecules display the desired antigenicity (e.g., of the type of cancer or pathogen).

### 4.6.1. OBTAINING ANTIGEN-PRESENTING CELLS

The antigen-presenting cells, including but not limited to macrophages, dendritic cells and B-cells, are preferably obtained by production *in vitro* from stem and progenitor cells from human peripheral blood or bone marrow as described by Inaba, K., *et al.*, 1992, J. Exp. Med. 176:1693-1702. Dendritic cells can be obtained by any of various methods known in the art. By way of example but not limitation, dendritic cells can be obtained by the methods described in Sallusto *et al.*, 1994, J Exp Med 179:1109-1118 and Caux *et al.*, 1992, Nature 360, 258-261 which are incorporated herein by reference in their entireties. In a preferred aspect, human dendritic cells obtained from human blood cells are used.

APC can be obtained by any of various methods known in the art. In one aspect, human macrophages are used, obtained from human blood cells. By way of example but not limitation, macrophages can be obtained as follows:

Mononuclear cells are isolated from peripheral blood of a patient (preferably the patient to be treated), by Ficoll-Hypaque gradient centrifugation and are seeded on tissue culture dishes which are pre-coated with the patient's own serum or with other AB+ human serum. The cells are incubated at 37°C for 1 hour, then non-adherent cells are removed by pipetting. To the adherent cells left in the dish, is added cold (4°C) 1 mM EDTA in phosphate-buffered saline and the dishes are left at room temperature for 15 minutes. The cells are harvested, washed with RPMI buffer and suspended in RPMI buffer. Increased numbers of macrophages may be obtained by incubating at 37°C with macrophage-colony stimulating factor (M-CSF).

### 4.6.2. SENSITIZATION OF MACROPHAGES AND ANTIGEN PRESENTING CELLS WITH HSP-PEPTIDE OR α2M-PEPTIDE COMPLEXES

APC are sensitized with HSP or α2M bound to antigenic peptides preferably by incubating the cells *in vitro* with the complexes. The APC are sensitized with complexes of HSPs or α2M and antigenic molecules by incubating *in vitro* with the HSP-complex or α2M-complex at 37°C for 15 minutes to 24 hours. By way of example but not limitation, $4x10^7$ dendritic cells can be incubated with 10 microgram gp96-peptide complexes per ml or 100 microgram HSP90-peptide complexes per ml at 37°C for 15 minutes-24 hours in 1 ml plain RPMI medium. The cells are washed three times and resuspended in a physiological medium preferably sterile, at a convenient concentration (*e.g.*, $1x10^7$/ml) for injection in a patient. Preferably, the patient into which the sensitized dendritic cells are injected is the patient from which the dendritic cells were originally isolated (autologous embodiment).

Optionally, the ability of sensitized APC to stimulate, for example, the antigen-specific, class I-restricted cytotoxic T-lymphocytes (CTL) can be monitored by their ability to stimulate CTLs to release tumor necrosis factor, and by their ability to act as targets of such CTLs.

### 4.6.3. REINFUSION OF SENSITIZED APC

The sensitized APCs are reinfused into the patient systemically, preferably intradermally, by conventional clinical procedures. These activated cells are reinfused, preferentially by systemic administration into the autologous patient. Patients generally receive from about $10^6$ to about $10^{12}$ sensitized dendritic cells depending on the condition of the patient. In some regimens, patients may optionally receive in addition a suitable dosage of a biological response modifier

including but not limited to the cytokines IFN-α, IFN-γ, IL-2, IL-4, IL-6, TNF or other cytokine growth factor.

## 4.7. PHARMACEUTICAL PREPARATIONS AND METHODS OF ADMINISTRATION

The complexes of antigenic proteins/peptides bound to HSPs and/or α2M prepared by the methods of the invention can be administered to a patient at therapeutically effective doses to treat or ameliorate a cell proliferative disorder or infectious disease. A therapeutically effective dose refers to that amount of the complexes sufficient to result in amelioration of symptoms of such a disorder. The effective dose of the complexes may be different when another treatment modality is being used in combination. The appropriate and recommended dosages, formulation and routes of administration for treatment modalities such as chemotherapeutic agents, radiation therapy and biological/immunotherapeutic agents such as cytokines are known in the art and described in such literature as the *Physician's Desk Reference* (56th ed., 2002).

### 4.7.1. EFFECTIVE DOSE

The compositions of the present invention, comprising an immunogenic, effective amount of complexes of a population of antigenic peptides with HSP and/or α2M are administered to a subject in need of treatment against cancer or an infectious disease, as a method of inducing an immune response against that cancer or infectious disease. Toxicity and therapeutic efficacy of such complexes can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the $LD_{50}$ (the dose lethal to 50% of the population) and the $ED_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio $LD_{50}/ED_{50}$. Complexes that exhibit large therapeutic indices are preferred. While complexes that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such complexes to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

In one embodiment, the data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of complexes lies preferably within a range of circulating concentrations that include the $ED_{50}$ with little or no

toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any complexes used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the $IC_{50}$ (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In another embodiment, an amount of hsp70- and/or gp96-antigenic molecule complexes is administered that is in the range of about 0.1 microgram to about 600 micrograms, and preferably about 1 micrograms to about 60 micrograms for a human patient. The amount of hsp70- and/or gp96 complexes administered is 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 250, 300, 400, 500 or 600 micrograms. Preferably, the amount is less than 100 micrograms. Most preferably, the amount of hsp70- and/or gp96 complexes administered is 5 micrograms, 25 micrograms, or 50 micrograms. The dosage for hsp-90 peptide complexes in a human patient provided by the present invention is in the range of about 5 to 5,000 micrograms. Preferably, the the amount of hsp90 complexes administered is 5, 10, 25, 50, 60, 70, 80, 90, 100, 200, 250, 500, 1000, 2000, 2500, or 5000 microgram, the most preferred dosage being 100 microgram. These doses are preferably administered intradermally or subcutaneously. These doses can be given once or repeatedly, such as daily, every other day, weekly, biweekly, or monthly. Preferably, the complexes are given once weekly for a period of about 4-6 weeks, and the mode or site of administration is preferably varied with each administration. Thus, by way of example and not limitation, the first injection may be given subcutaneously on the left arm, the second on the right arm, the third on the left belly, the fourth on the right belly, the fifth on the left thigh, the sixth on the right thigh, etc. The same site may be repeated after a gap of one or more injections. Also, split injections may be given. Thus, for example, half the dose may be given in one site and the other half on an other site on the same day. Alternatively, the mode of administration is sequentially varied, *e.g.*, weekly injections are given in sequence intradermally, intramuscularly, subcutaneously, intravenously or intraperitoneally. Preferably, the once weekly dose is given for a period of 4 weeks. After 4-6 weeks, further injections are preferably given at two-week intervals over a period of time of one or more months, or until supply of complexes is

exhausted. Later injections may be given monthly. The pace of later injections may be modified, depending upon the patient's clinical progress and responsiveness to the immunotherapy. In a preferred example, intradermal administrations are given, with each site of administration varied sequentially.

Accordingly, the invention provides methods of preventing and treating cancer or an infectious disease in a subject comprising administering a composition which stimulates the immunocompetence of the host individual and elicits specific immunity against the preneoplastic and/or neoplastic cells or infected cells.

In a specific embodiment, during combination therapy, the HSP complexes is administered in a sub-optimal amount, *e.g.*, an amount that does not manifest detectable therapeutic benefits when administered in the absence of the therapeutic modality, as determined by methods known in the art. In such methods, the administration of such a sub-optimal amount of HSP complexes to a subject receiving a therapeutic modality results in an overall improvement in effectiveness of treatment. In another specific embodiment, the α2M complexes is administered in a sub-optimal amount during combination therapy. In such methods, the administration of such a sub-optimal amount of α2M complexes to a subject receiving a therapeutic modality results in an overall improvement in effectiveness of treatment.

In a preferred embodiment, an HSP complexes is administered in an amount that does not result in tumor regression or cancer remission or an amount wherein the cancer cells have not been significantly reduced or have increased when said HSP complexes is administered in the absence of the therapeutic modality. In a preferred embodiment, the sub-optimal amount of HSP complexes is administered to a subject receiving a treatment modality whereby the overall effectiveness of treatment is improved. In another preferred embodiment, an α2M complexes is administered in an amount that does not result in tumor regression or cancer remission or an amount wherein the cancer cells have not been significantly reduced or have increased when said α2M complexes is administered in the absence of the therapeutic modality. In a preferred embodiment, the sub-optimal amount of α2M complexes is administered to a subject receiving a treatment modality whereby the overall effectiveness of treatment is improved. Among these subjects being treated with HSP or α2M complexes are those receiving chemotherapy or radiation therapy. A sub-optimal amount can be determined by appropriate animal studies. Such a sub-optimal amount in humans can be determined by extrapolation from experiments in

animals.

In certain specific embodiments, an HSP or α2M complexes is administered to a subject already receiving a chemotherapeutic agent, such as Gleevec™ (*e.g.*, 400-800 mg daily in capsule form, 400-600 mg doses administered once daily, or 800 mg dose administered daily in two doses of 400 mg each). Gleevec™ is used hereinbelow as a non-limiting example of a chemotherapeutic agent that can be used in combination. For many other chemotherapeutic agents, a similar dosing regime can be used. In such embodiments, the appropriate HSP/α2M complexes is initially administered to a subject who has already been receiving Gleevec™ in the absence of HSP/α2M complexes 2 days, 2 days to 1 week, 1 week to 1 month, 1 month to 6 months, 6 months to 1 year prior to administration of HSP/α2M complexes in addition to Gleevec™. In a specific embodiment, HSP/α2M complexes are administered to a subject wherein the subject showed resistance to treatment with Gleevec™ alone.

In other embodiments, HSP/α2M complexes are initially administered to a subject concurrently with the initial administration of Gleevec™.

In yet other specific embodiments, Gleevec™ (*e.g.*, 400-800 mg daily in capsule form) is administered to a subject already receiving treatment comprising administration of HSP/α2M complexes. In such embodiments, Gleevec™ is initially administered to a subject who has already been receiving HSP/α2M complexes in the absence of Gleevec™ 2 days, 2 days to 1 week, 1 week to 1 month, 1 month to 6 months, 6 months to 1 year prior to administration of Gleevec™ in addition to administration of HSP/α2M complexes.

In a specific embodiment, a chemotherapeutic agent such as Gleevec™ is administered orally. In another specific embodiment, the HSP/α2M complexes are administered intradermally.

In each of the methods contemplated above, the subject, by way of example, receives 50 mg to 100 mg, 100 mg to 200 mg, 200 mg to 300 mg, 300 mg to 400 mg, 400 mg to 500 mg, 500 mg to 600 mg, 600 mg to 700 mg, 700 mg to 800 mg, 800 mg to 900 mg, or 900 mg to 1000 mg of chemotherapeutic agents, such as Gleevec™ , daily. In certain embodiments, the total daily dose is administered to a subject as two daily doses of 25mg to 50 mg, 50 mg to 100 mg, 100 mg to 200 mg, 200 mg to 300 mg, 300 mg to 400 mg, or 400 mg to 500 mg.

### 4.7.2. THERAPEUTIC REGIMENS

For any of the combination therapies described above for treatment or prevention of

cancer and infectious diseases, the complexes of the invention can be administered prior to, concurrently with, or subsequent to the administration of the non-HSP and non-α2M based modality. The non-HSP and non-α2M based modality can be any one of the modalities described above for treatment or prevention of cancer or infectious disease.

In one embodiment, the complexes of the invention is administered to a subject at reasonably the same time as the other modality. This method provides that the two administrations are performed within a time frame of less than one minute to about five minutes, or up to about sixty minutes from each other, for example, at the same doctor's visit.

In another embodiment, the complexes of the invention and a modality are administered at exactly the same time. In yet another embodiment the complexes of the invention and the modality are administered in a sequence and within a time interval such that the complexes of the invention and the modality can act together to provide an increased benefit than if they were administered alone. In another embodiment, the complexes of the invention and a modality are administered sufficiently close in time so as to provide the desired therapeutic or prophylactic outcome. Each can be administered simultaneously or separately, in any appropriate form and by any suitable route. In one embodiment, the complexes of the invention and the modality are administered by different routes of administration. In an alternate embodiment, each is administered by the same route of administration. The complexes of the invention can be administered at the same or different sites, *e.g.* arm and leg. When administered simultaneously, the complexes of the invention and the modality may or may not be administered in admixture or at the same site of administration by the same route of administration.

In a preferred embodiment, the complexes of the invention are administered according to the regimen described in Section 4.7.1. In various embodiments, the complexes of the invention and the modality are administered less than 1 hour apart, at about 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, no more than 24 hours apart or no more than 48 hours apart. In other embodiments, the complexes of the invention and vaccine composition are administered 2 to 4 days apart, 4 to 6 days apart, 1 week a part, 1 to 2 weeks apart, 2 to 4 weeks apart, one moth apart, 1 to 2 months apart, or 2 or more months apart. In preferred embodiments, the complexes of the invention and the modality are

administered in a time frame where both are still active. One skilled in the art would be able to determine such a time frame by determining the half life of each administered component.

In one embodiment, the complexes of the invention and the modality are administered within the same patient visit. In a specific preferred embodiment, the complexes of the invention is administered prior to the administration of the modality. In an alternate specific embodiment, the complexes of the invention is administered subsequent to the administration of the modality.

In certain embodiments, the complexes of the invention and the modality are cyclically administered to a subject. Cycling therapy involves the administration of the complexes of the invention for a period of time, followed by the administration of a modality for a period of time and repeating this sequential administration. Cycling therapy can reduce the development of resistance to one or more of the therapies, avoid or reduce the side effects of one of the therapies, and/or improve the efficacy of the treatment. In such embodiments, the invention contemplates the alternating administration of a complexes of the invention followed by the administration of a modality 4 to 6 days later, preferable 2 to 4 days, later, more preferably 1 to 2 days later, wherein such a cycle may be repeated as many times as desired. In certain embodiments, the complexes of the invention and the modality are alternately administered in a cycle of less than 3 weeks, once every two weeks, once every 10 days or once every week. In a specific embodiment, complexes of the invention is administered to a subject within a time frame of one hour to twenty four hours after the administration of a modality. The time frame can be extended further to a few days or more if a slow- or continuous-release type of modality delivery system is used.

### 4.7.3. FORMULATIONS AND USE

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the complexes and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) oral, buccal, parenteral, rectal, or transdermal administration. Non-invasive methods of administration are also contemplated.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable

excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active complexes.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the complexes for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the complexes and a suitable powder base such as lactose or starch.

The complexes may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a

suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The complexes may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the complexes may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the complexes may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Also encompassed is the use of adjuvants in combination with or in admixture with the complexes of the invention. Adjuvants contemplated include but are not limited to mineral salt adjuvants or mineral salt gel adjuvants, particulate adjuvants, microparticulate adjuvants, mucosal adjuvants, and immunostimulatory adjuvants, such as those described in Section 4.5. Adjuvants can be administered to a subject as a mixture with complexes of the invention, or used in combination with the complexes as described in Section 4.7.2.

Also contemplated is the use of adenosine diphosphate (ADP) in combination with or in admixture with the complexes of the invention, preferably gp96 complexes.

#### 4.7.4. KITS

The invention also provides kits for carrying out the methods and/or therapeutic regimens of the invention.

In one embodiment, such kits comprise in one or more containers protein preparations comprising antigenic proteins and peptides for combining with HSPs and/or α2M that are provided in a second container. In another embodiment, such kits comprise in one or more containers digested peptides comprising antigenic peptides for combining with HSPs and/or α2M that are provided in a second container. Alternatively, proteins and/or peptides can be supplied in one or more containers for complexing to HSPs and/or α2M isolated from a specific patient for

autologous administration. Optionally, a purified HSP for complexing to proteins and peptides is further provided in a second container.

In another embodiment, such kits comprise in one or more containers therapeutically or prophylactically effective amounts of the complexed proteins/peptides to HSPs and/or α2M, preferably purified, in pharmaceutically acceptable form. The kits optionally further comprise in a second container sensitized APCs, preferably purified.

The HSP or α2M complexes in a container of a kit of the invention may be in the form of a pharmaceutically acceptable solution, *e.g.*, in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the HSP and α2M complexes may be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (*e.g.*, saline, dextrose solution, etc.), preferably sterile, to reconstitute the HSPs and α2M or α2M and HSP-containing complexes to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the HSP and α2M complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of α2M and HSP-peptide complexes by a clinician or by the patient.

Kits are also provided for carrying out the combination therapies of the present invention. In one embodiment, a kit comprises a first container containing a purified HSP complexes or α2M prepration and a second container containing a non-HSP and non-α2M based therapeutic modality for treatment of cancer. Preferably, the cancer is CML, the HSP complexes comprises hsp70-peptide complexes, and the therapeutic modality is Gleevec™. In a specific embodiment, the second container contains imatinib mesylate. In another specific embodiment, the imatinib mesylate is purified.

In a specific embodiment, a kit comprises a first container containing a purified HSP complexes or α2M complexes in an amount ineffective to treat a disease or disorder when administered alone; and a second container containing a non-HSP and non-α2M based treatment modality in an amount that, when administered before, concurrently with, or after the administration of the HSP complexes or α2M complexes in the first container, is effective to improve overall treatment effectiveness over the effectiveness of the administration of each component alone. In another specific embodiment, a kit comprises a first container containing a

purified HSP complexes or α2M complexes in an amount ineffective to treat a disease or disorder when administered alone; and a second container containing one or more non-HSP and non-α2M based treatment modalities in an amount that, when administered before, concurrently with, or after the administration of the HSP complexes or α2M complexes in the first container, is effective to improve overall treatment effectiveness over the effectiveness of the administration of the HSP complexes or α2M complexes administered alone or the treatment modalities administered alone. In yet another specific embodiment, a first container containing a purified HSP complexes or α2M complexes in an amount ineffective to treat a disease or disorder when administered alone; and a second container and third container, each containing a non-HSP and non-α2M based treatment modality in an amount that, when administered before, concurrently with, or after the administration of the HSP complexes or α2M complexes in the first container, is effective to improve overall treatment effectiveness over the effectiveness of the administration of HSP complexes or α2M complexes administered alone or treatment modalities administered alone. In a preferred specific embodiment, the invention provides a kit comprising in a first container, a purified HSP complexes or α2M comprising a population of noncovalent HSP-peptide complexes or α2M-peptide complexes of the invention; in a second container, a composition comprising an anti-cancer agent; and in a third container, a composition comprising a cytokine or an adjuvant.

The kit may for example comprise metal or plastic foil, such as a blister pack. The kit may be accompanied by one or more reusable or disposable device(s) for administration (e.g, syringes, needles, dispensing pens) and/or instructions for administration.

## 4.8. DETERMINATION OF IMMUNOGENICITY OF THE HSP AND α2M COMPLEXES

Optionally, the HSP-protein complexes, HSP-peptide complexes, α2M-protein complexes and α2M-peptide complexes of the invention can be assayed for immunogenicity using any method known in the art. By way of example but not limitation, one of the following procedures can be used. In a preferred embodiment, the ELISPOT assay is used (see, *infra*, Section 4.9.4).

### 4.8.1. THE MLTC ASSAY

Briefly, mice are injected with an amount of the HSP- and/or α2M complexes, using any convenient route of administration. As a negative control, other mice are injected with, *e.g.*, HSP complexed to proteins and/or peptides prepared from normal tissue. Cells known to contain specific antigens, *e.g.* tumor cells or cells infected with an agent of an infectious disease, may act as a positive control for the assay. The mice are injected twice, 7-10 days apart. Ten days after the last immunization, the spleens are removed and the lymphocytes released. The released lymphocytes may be re-stimulated subsequently *in vitro* by the addition of dead cells that expressed the antigen of interest.

For example, $8x10^6$ immune spleen cells may be stimulated with $4x10^4$ mitomycin C treated or γ-irradiated (5-10,000 rads) cells containing the antigen of interest (or cells transfected with an appropriate gene, as the case may be) in 3ml RPMI medium containing 10% fetal calf serum. In certain cases 33% secondary mixed lymphocyte culture supernatant may be included in the culture medium as a source of T cell growth factors (*See,* Glasebrook, et al., 1980, *J. Exp. Med.* 151:876). To test the primary cytotoxic T cell response after immunization, spleen cells may be cultured without stimulation. In some experiments spleen cells of the immunized mice may also be re-stimulated with antigenically distinct cells, to determine the specificity of the cytotoxic T cell response.

Six days later the cultures are tested for cytotoxicity in a 4 hour $^{51}Cr$-release assay (*See*, Palladino, et al., 1987, *Cancer Res.* 47:5074-5079 and Blachere, at al., 1993, *J. Immunotherapy* 14:352-356). In this assay, the mixed lymphocyte culture is added to a target cell suspension to give different effector:target (E:T) ratios (usually 1:1 to 40:1). The target cells are prelabelled by incubating $1x10^6$ target cells in culture medium containing 20 mCi $^{51}Cr$/ml for one hour at 37°C. The cells are washed three times following labeling. Each assay point (E:T ratio) is performed in triplicate and the appropriate controls incorporated to measure spontaneous $^{51}Cr$ release (no lymphocytes added to assay) and 100% release (cells lysed with detergent). After incubating the cell mixtures for 4 hours, the cells are pelletted by centrifugation at 200g for 5 minutes. The amount of $^{51}Cr$ released into the supernatant is measured by a gamma counter. The percent cytotoxicity is measured as cpm in the test sample minus spontaneously released cpm divided by the total detergent released cpm minus spontaneously released cpm.

In order to block the MHC class I cascade a concentrated hybridoma supernatant derived from K-44 hybridoma cells (an anti-MHC class I hybridoma) is added to the test samples to a

final concentration of 12.5%.

### 4.8.2. CD4+ T-CELL PROLIFERATION ASSAY

Primary T cells are obtained from spleen, fresh blood, or CSF and purified by centrifugation using FICOLL-PAQUE PLUS (Pharmacia, Upsalla, Sweden) essentially as described by Kruse and Sebald, 1992, EMBO J. 11: 3237-3244. The peripheral blood mononuclear cells are incubated for 7-10 days with a lysate of cells expressing an antigenic molecule. Antigen presenting cells may, optionally be added to the culture 24 to 48 hours prior to the assay, in order to process and present the antigen in the lysate. The cells are then harvested by centrifugation, and washed in RPMI 1640 media (GibcoBRL, Gaithersburg, Md.). $5x10^4$ activated T cells/well are in RPMI 1640 media containing 10% fetal bovine serum, 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulphate in 96 well plates for 72 hrs at 37°C., pulsed with 1 µCi $^3$H-thymidine (DuPont NEN, Boston, Mass.)/well for 6 hrs, harvested, and radioactivity measured in a TOPCOUNT scintillation counter (Packard Instrument Co., Meriden, Conn.).

### 4.8.3. ANTIBODY RESPONSE ASSAY

In a certain embodiment of the invention, the immunogenicity of an HSP- or α2M-complex is determined by measuring antibodies produced in response to the vaccination with the complex. In one mode of the embodiment, microtitre plates (96-well Immuno Plate II, Nunc) are coated with 50 µl/well of a 0.75 µg/ml solution of a purified, non-HSP- or α2M- complexed form of the proteins/peptides used in the vaccine in PBS at 4°C for 16 hours and at 20°C for 1 hour. The wells are emptied and blocked with 200 µl PBS-T-BSA (PBS containing 0.05% (v/v) TWEEN 20 and 1% (w/v) bovine serum albumin) per well at 20°C for 1 hour, then washed 3 times with PBS-T. Fifty µl/well of plasma or CSF from a vaccinated animal (such as a model mouse or a human patient) is applied at 20°C for 1 hour, and the plates are washed 3 times with PBS-T. The anti-peptide antibody activity is then measured calorimetrically after incubating at 20°C for 1 hour with 50µl/well of sheep anti-mouse or anti-human immunoglobulin, as appropriate, conjugated with horseradish peroxidase (Amersham) diluted 1:1,500 in PBS-T-BSA and (after 3 further PBS-T washes as above) with 50 µl of an o-phenylene diamine (OPD)-$H_2O_2$ substrate solution. The reaction is stopped with 150 µl of 2M $H_2SO_4$ after 5 minutes and

absorbance is determined in a Kontron SLT-210 photometer (SLT Lab-instr., Zurich, Switzerland) at 492 nm (ref. 620 nm).

### 4.8.4. CYTOKINE DETECTION ASSAY

The CD4+ T cell proliferative response to HSP- or α2M-complexes of the invention may be measured by detection and quantitation of the levels of specific cytokines. In one embodiment, for example, intracellular cytokines may be measured using an IFN-γ detection assay to test for immunogenicity of a complex of the invention. In an example of this method, peripheral blood mononuclear cells from a subject treated with a HSP-peptide or α2M peptide complex are stimulated with peptide antigens of a given tumor or with peptide antigens of an agent of infectious disease. Cells are then stained with T cell-specific labeled antibodies detectable by flow cytometry, for example FITC-conjugated anti-CD8 and PerCP-labeled anti-CD4 antibodies. After washing, cells are fixed, permeabilized, and reacted with dye-labeled antibodies reactive with human IFN-γ (PE- anti-IFN-γ). Samples are analyzed by flow cytometry using standard techniques.

Alternatively, a filter immunoassay, the enzyme-linked immunospot assay (ELISPOT) assay, may be used to detect specific cytokines surrounding a T cell. In one embodiment, for example, a nitrocellulose-backed microtiter plate is coated with a purified cytokine-specific primary antibody, *i.e.,* anti-IFN-γ, and the plate is blocked to avoid background due to nonspecific binding of other proteins. A sample of mononuclear blood cells, containing cytokine-secreting cells, obtained from a subject treated with a HSP-peptide and/or α2M peptide complex, which sample is diluted onto the wells of the microtitre plate. A labeled, *e.g.*, biotin-labeled, secondary anti-cytokine antibody is added. The antibody cytokine complex can then be detected, *i.e.* by enzyme-conjugated streptavidin – cytokine-secreting cells will appear as "spots" by visual, microscopic, or electronic detection methods.

### 4.8.5. TETRAMER ASSAY

In another embodiment, the "tetramer staining" assay (Altman et al., 1996, Science 274: 94-96) may be used to identify antigen-specific T-cells. For example, in one embodiment, an MHC molecule containing a specific peptide antigen, such as a tumor-specific antigen, is multimerized to make soluble peptide tetramers and labeled, for example, by complexing to

streptavidin. The MHC-peptide antigen complex is then mixed with a population of T cells obtained from a subject treated with a HSP- or α2M-complex. Biotin is then used to stain T cells which express the antigen of interest, *i.e.*, the tumor-specific antigen.

## 4.9. MONITORING OF EFFECTS DURING CANCER PREVENTION AND IMMUNOTHERAPY

The effect of immunotherapy with HSP- or α2M-complexes on the development and progression of neoplastic diseases can be monitored by any method known to one skilled in the art, including but not limited to measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes *in vitro*; c) levels of tumor specific antigens, *e.g.*, carcinoembryonic (CEA) antigens; d) changes in the morphology of tumors using techniques such as a computed tomographic (CT) scan; and e) changes in levels of putative biomarkers of risk for a particular cancer in individuals at high risk, and f) changes in the morphology of tumors using a sonogram.

The following subsections describe optional, exemplary procedures.

### 4.9.1. DELAYED HYPERSENSITIVITY SKIN TEST

Delayed hypersensitivity skin tests are of great value in the overall immunocompetence and cellular immunity to an antigen. Inability to react to a battery of common skin antigens is termed anergy (Sato, T., *et al.*, 1995, *Clin. Immunol. Pathol.* 74:35-43).

Proper technique of skin testing requires that the antigens be stored sterile at 4°C, protected from light and reconstituted shortly before use. A 25- or 27-gauge need ensures intradermal, rather than subcutaneous, administration of antigen. Twenty-four and 48 hours after intradermal administration of the antigen, the largest dimensions of both erythema and induration are measured with a ruler. Hypoactivity to any given antigen or group of antigens is confirmed by testing with higher concentrations of antigen or, in ambiguous circumstances, by a repeat test with an intermediate test.

### 4.9.2. ACTIVITY OF CYTOLYTIC T-LYMPHOCYTES *IN VITRO*

$8x10^6$ Peripheral blood derived T lymphocytes isolated by the Ficoll-Hypaque centrifugation gradient technique, are restimulated with $4x10^4$ mitomycin C treated tumor cells in

3ml RPMI medium containing 10% fetal calf serum. In some experiments, 33% secondary mixed lymphocyte culture supernatant or IL-2, is included in the culture medium as a source of T cell growth factors.

In order to measure the primary response of cytolytic T-lymphocytes after immunization, T cells are cultured without the stimulator tumor cells. In other experiments, T cells are restimulated with antigenically distinct cells. After six days, the cultures are tested for cytotoxicity in a 4 hour $^{51}$Cr-release assay. The spontaneous $^{51}$Cr-release of the targets should reach a level less than 20%. For the anti-MHC class I blocking activity, a tenfold concentrated supernatant of W6/32 hybridoma is added to the test at a final concentration of 12.5% (Heike M., *et al.*, *J. Immunotherapy* 15:165-174).

### 4.9.3. LEVELS OF TUMOR SPECIFIC ANTIGENS

Although it may not be possible to detect unique tumor antigens on all tumors, many tumors display antigens that distinguish them from normal cells. The monoclonal antibody reagents have permitted the isolation and biochemical characterization of the antigens and have been invaluable diagnostically for distinction of transformed from nontransformed cells and for definition of the cell lineage of transformed cells. The best-characterized human tumor-associated antigens are the oncofetal antigens. These antigens are expressed during embryogenesis, but are absent or very difficult to detect in normal adult tissue. The prototype antigen is carcinoembryonic antigen (CEA), a glycoprotein found on fetal gut an human colon cancer cells, but not on normal adult colon cells. Since CEA is shed from colon carcinoma cells and found in the serum, it was originally thought that the presence of this antigen in the serum could be used to screen patients for colon cancer. However, patients with other tumors, such as pancreatic and breast cancer, also have elevated serum levels of CEA. Therefore, monitoring the fall and rise of CEA levels in cancer patients undergoing therapy has proven useful for predicting tumor progression and responses to treatment.

Several other oncofetal antigens have been useful for diagnosing and monitoring human tumors, *e.g.*, alpha-fetoprotein, an alpha-globulin normally secreted by fetal liver and yolk sac cells, is found in the serum of patients with liver and germinal cell tumors and can be used as a matter of disease status.

### 4.9.4. COMPUTED TOMOGRAPHIC (CT) SCAN

CT remains the choice of techniques for the accurate staging of cancers. CT has proved more sensitive and specific than any other imaging techniques for the detection of metastases.

### 4.9.5. MEASUREMENT OF PUTATIVE BIOMARKERS

The levels of a putative biomarker for risk of a specific cancer are measured to monitor the effect of compositions comprising cytosolic and membrane-derived proteins. For example, in individuals at enhanced risk for prostate cancer, serum prostate-specific antigen (PSA) is measured by the procedure described by Brawer, M.K., *et al.*, 1992, J. Urol. 147:841-845, and Catalona, W.J., *et al.*, 1993, JAMA 270:948-958; or in individuals at risk for colorectal cancer CEA is measured as described above in Section 4.5.3; and in individuals at enhanced risk for breast cancer, 16-α-hydroxylation of estradiol is measured by the procedure described by Schneider, J. *et al.*, 1982, Proc. Natl. Acad. Sci. ISA 79:3047-3051. The references cited above are incorporated by reference herein in their entirety.

### 4.9.6. SONOGRAM

A Sonogram remains an alternative choice of technique for the accurate staging of cancers.

## 5. EXAMPLE

The following experiment demonstrates that complexes of (a) antigenic peptides derived from a cellular fraction, with (b) either HSP or alpha-2-macroglobulin (α2M), are effective at protecting an animal prophylactically from cancer cell growth.

## 5.1. MATERIALS AND METHOD

### 5.1.1 Protein purification.

For purification of α2M, serum from mice was diluted 1:1 with 0.04M Tris pH 7.6, 0.15M NaCl and applied to a 65ml Sephacryl S 300R (SIGMA) column equilibrated and eluted with the same buffer. α2M-positive fractions were determined by a dot-blot and the buffer in the fraction was changed to a 0.01M sodium phosphate buffer pH 7.5 by use of a PD-10 column. The protein-containing fractions were applied to a Concanavalin A sepharose column. Bound protein was eluted with 0.2M methylmannose pyranoside and applied to a DEAE column equilibrated with 0.05M sodium acetate buffer. α2M was eluted in a pure form as analyzed by SDS-PAGE and immunoblotting with 0.13M sodium acetate.

In some experiments, α2M was purchased from SIGMA.

Gp96 was obtained by the method described in Section 4.3.3.

### 5.1.2 Tumor rejection assays

All immunizations were done intradermally in 100μl volume of PBS. Two immunizations were given one week apart. Seven micrograms of α2M or 1μg of gp96 was used per injection either as a complex or alone. Live tumor cells (100,000) were washed free of culture medium, resuspended in PBS and injected intradermally one week after the last immunization. Tumors were measured in two dimensions. Half of the average was used as the radius of the tumor to calculate the tumor volume. P values were determined using single-classification analysis of variance (ANOVA).

### 5.1.3 Generation of complexes.

Cell lysate was obtained from live Meth A cells by dounce homogenization followed by ultracentrifugation. 100,000g supernatant was treated with 0.1% trifluoroacetic acid (TFA) and 3mM ATP for 10 hours followed by centrifugation in a CENTRICON membrane filter (Millipore) with a 10kDa cut off limit. Peptides less than10kDa (referred to as "MethA10") were further isolated by binding to a C18 reverse phase column, eluting the peptides with methanol, drying the peptides down in a vacuum, and reconstituting the peptides in a buffer suitable for complexing. Gp96, α2M, or albumin (which was used as a control) was heated to 50°C in the presence of 50 molar excess of MethA10. The reactions containing the resulting complexes were placed at room temperature for 30

minutes and then placed on ice. Free, uncomplexed peptide was removed using CENTRICON 50 (Millipore). Complexes thus made were used for immunizations.

## 5.2. RESULTS

In this experiment, the Meth A tumor model was used to demonstrate the anti-tumor immunity elicited by gp96-peptide complexes, and α2M-peptide complexes. The antigenic MHC I epitopes of this tumor are unknown. Meth A cell lysates were treated with ATP and trifluoroacetic acid (TFA) and the fraction of peptides that were less than 10 kD (MethA10) was collected and complexed to α2M or gp96 as described above. BALB/c mice were immunized with α2M or gp96, un-complexed or complexed with MethA10. BALB/c mice were also immunized with albumin-MethA10 or PBS as negative controls. Immunizations were done twice, one week apart. All mice were challenged intradermally with 100,000 live Meth A cells one week after the last immunization. Tumor growth was monitored every 5 days up to day 20 after the challenge.

Table 1

| Compositions used in immunization of mice | Number of mice challenged with tumor cells at day 0 | Number of mice with measurable tumor at day 20 |
|---|---|---|
| MethA10 only | 5 | 5 |
| Albumin-MethA10 | 5 | 5 |
| PBS | 5 | 5 |
| α2M-MethA10 complexes | 5 | 0 |
| Gp96-MethA10 complexes | 5 | 0 |
| Gp96 purified from liver | 5 | 5 |
| α2M purified from serum | 5 | 4 |

The data in Table 1 shows significant tumor protection in mice immunized with α2M-MethA10 ($p<0.05$) or gp96-MethA10 ($p<0.05$) complexes but not mice immunized with α2M alone, gp96 alone, albumin-MethA10 or PBS.

## 5.3. DISCUSSION

The experiment on immunization against tumors described herein demonstrates a novel approach to immunotherapy of cancers, whereby an array of total cellular peptides from the tumor, including self and antigenic peptides, is complexed to an HSP or α2M. Such complexes effectively stimulated the host's immune system to respond specifically as shown herein. The data indicate that the utility of this approach in prophylaxis can be extended to treatment of pre-existing disease, as well as in treatment and prevention of pathogenic infections.

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims along with the full scope of equivalents to which such claims are entitled.

**WHAT IS CLAIMED IS:**

1. A method of treating or preventing a type of cancer, comprising

administering to a subject in need of such treatment or prevention a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic proteins wherein said population of complexes were produced by complexing heat shock protein or alpha-2-macroglobulin to (i) antigenic proteins that are at least 50% of the different proteins present in the cells of said type of cancer, or (ii) at least 50 different proteins present in the cells of said type of cancer; and

administering to said subject at least one treatment modality that does not comprise a heat shock protein or alpha-2-macroglobulin.

2. A method of treating or preventing a type of cancer, comprising

administering to a subject in need of such treatment or prevention a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic peptides wherein said population of complexes were produced by a method comprising digesting a protein preparation comprising (i) at least 50% of the different proteins present in cells of said type of cancer or (ii) at least 50 different proteins present in cells of said type of cancer with one or more proteases to produce a population of antigenic peptides, and complexing the population of antigenic peptides to heat shock protein or alpha-2-macroglobulin; and

administering to said subject at least one treatment modality that does not comprise a heat shock protein or alpha-2-macroglobulin.

3. A method of treating or preventing a type of cancer, comprising

administering to a subject in need of such treatment or prevention a composition comprising a population of complexes, said complexes comprising (i) heat shock protein and/or alpha-2-macroglobulin and (ii) antigenic peptides wherein said population of complexes were produced by a method comprising (a) exposing a protein preparation comprising (A) at least 50% of the different proteins present in cells of said type of cancer or (B) at least 50 different proteins present in cells of said type of cancer to ATP, guanidium hydrochloride, and/or acidic conditions, to produce a

population of antigenic peptides; (b) recovering the population of antigenic peptides; and (c) complexing the population of antigenic peptides to heat shock protein or alpha-2-macroglobulin; and

administering to said subject at least one treatment modality that does not comprise a heat shock protein or alpha-2-macroglobulin.

4. A method of treating or preventing a type of infectious disease, comprising

administering to a subject in need of such treatment or prevention a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic proteins wherein said population of complexes were produced by complexing heat shock protein or alpha-2-macroglobulin to antigenic proteins that are at least 50% of the different proteins or at least 50 different proteins present in antigenic cells, a cellular fraction thereof, or viral particles that express an antigenic determinant of an agent that causes the infectious disease; and

administering to said subject at least one treatment modality that does not comprise a heat shock protein or alpha-2-macroglobulin.

5. A method of treating or preventing a type of infectious disease, comprising

administering to a subject in need of such treatment or prevention a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic peptides wherein said population of complexes were produced by a method comprising (i) digesting a protein preparation comprising at least 50% of the different proteins or at least 50 different proteins present in antigenic cells, a cellular fraction thereof or viral particles that express an antigenic determinant of an agent that causes the infectious disease with either a protease or a plurality of different proteases; and (ii) complexing the population of antigenic peptides to heat shock protein or alpha-2-macroglobulin; and

administering to said subject at least one treatment modality that does not comprise a heat shock protein or alpha-2-macroglobulin.

6. A method of treating or preventing a type of infectious disease, comprising

administering to a subject in need of such treatment or prevention a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic peptides wherein said complexes were produced by a method comprising (i) exposing a protein preparation comprising at least 50% of the different proteins or at least 50 different proteins present in antigenic cells, a cellular fraction thereof, or viral particles that express an antigenic determinant of an agent that causes the infectious disease to ATP, guanidium hydrochloride, and/or acidic conditions, to produce a population of antigenic peptides; (ii) recovering the population of antigenic peptides; and (iii) complexing the population of antigenic peptides to heat shock protein or alpha-2-macroglobulin; and

administering to said subject at least one treatment modality that does not comprise a heat shock protein or alpha-2-macroglobulin.

7. The method of claim 1 wherein said complexing the population of antigenic proteins to the heat shock proteins is via formation of a covalent bond.

8. The method of claim 1 wherein said complexing the population of antigenic proteins to the heat shock proteins is via formation of a non-covalent bond.

9. The method of claim 2 or 3 wherein said complexing the population of antigenic peptides to the heat shock proteins is via formation of a covalent bond.

10. The method of claim 2 or 3 wherein said complexing the population of antigenic peptides to the heat shock proteins is via formation of a non-covalent bond.

11. The method of claim 4 wherein said complexing the population of antigenic proteins to α-2-macroglobulin is via formation of a covalent bond.

12. The method of claim 4 wherein said complexing the population of antigenic proteins to α-2-macroglobulin is via formation of a non-covalent bond.

13. The method of claim 5 or 6 wherein said complexing the population of antigenic peptides to the α-2-macroglobulin is via formation of a covalent bond.

14. The method of claim 5 or 6 wherein said complexing the population of antigenic peptides to α-2-macroglobulin is via formation of a non-covalent bond.

15. The method of claim 1 wherein said population of complexes comprising heat shock protein and/or alpha-2-macroglobulin, and antigenic proteins is purified.

16. The method of claim 4 wherein said population of complexes is purified.

17. The method of claim 2 or 3 wherein said population of complexes is purified.

18. The method of claim 5 or 6 wherein said population of complexes is purified.

19. The method of claim 1, 2 or 3, wherein the cells of same type of cancer are from a metastasis.

20. The method of claim 1, 2 or 3, wherein the cancer treated or prevented is a metastasis.

21. The method of claim 5, 6 or 7, wherein the antigenic cells are infected by the agent that causes the infectious disease.

22. The method of claim 5, 6 or 7, wherein the antigenic cells are infected by a variant of said agent, that displays antigenicity of said agent.

23. The method of claim 1, 2, or 3 wherein the at least treatment modality comprises a chemotherapeutic agent, an anti-angiogenic agent, a cytokine, a biological response modifier, a hormone, an antibody, a polynucleotide, an immunostimulatory oligonucleotide, a photodynamic therapeutic agent or radiation.

24. The method of claim 4, 5, or 6 wherein the at least one treatment modality comprises an antibiotic, an antiviral, an antiprotozoal compound, an antifungal compound, an antihelminthic compound, an antibody, a cytokine, a hormone, an immunostimulatory oligonucleotide, or a polynucleotide.

25. The method of claim 1, 2, 3, 4, 5, or 6 wherein said composition is administered before, concurrently with, or after administration of the at least one treatment modality.

26. The method of claim 1, 2, 3, 4, 5 or 6 wherein the subject has previously been non-responsive to treatment with said at least one treatment modality in the absence of said composition.

27. The method of claim 1, 2, 3, 4, 5, or 6 wherein said administering of said composition is repeated at weekly intervals.

28. The method of claim 1, 2, 3, 4, 5, or 6 wherein said administering of said composition is repeated at the same site of the subject.

29. The method of claim 1, 2, 3, 4, 5, or 6 wherein said administering of said composition is intradermally or subcutaneously.

30. The method of claim 1, 2, 3, 4, 5, or 6 wherein a sub-optimal amount of said composition is administered.

31. The method of claim 1, 2, 3, 4, 5, or 6 wherein a sub-optimal amount of said at least one treatment modality is administered.

32. The method of claim 1, 2, 3, 4, 5, or 6 wherein the subject is human.

33. The method of claim 1 wherein the antigenic proteins are autologous to the subject.

34. The method of claim 4 wherein the antigenic proteins are autologous to the subject.

35. The method of claim 2 or 3 wherein the antigenic peptides are autologous to the subject.

36. The method of claim 5 or 6 wherein the antigenic peptides are autologous to the subject.

37. A kit comprising

a first container containing a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic proteins, wherein said population of complexes were produced by complexing heat shock protein or alpha-2-macroglobulin to antigenic proteins that are at least 50% of the different proteins present in antigenic cells or at least 50 different proteins present in antigenic cells; and

a second container containing a treatment modality that does not comprise heat shock protein or alpha-2-macroglobulin.

38. A kit comprising

a first container containing a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic proteins, wherein said population of complexes were produced by a method comprising (i) digesting a protein preparation comprising at least 50% of the different proteins or at least 50 different proteins present in antigenic cells with one or more proteases to produce a population of antigenic peptides, and (ii) complexing the population of antigenic peptides to heat shock protein or alpha-2-macroglobulin; and

a second container containing a non-heat shock protein and non-alpha-2-macroglobulin-based treatment modality.

39. A kit comprising

a first container containing a composition comprising a population of complexes, said complexes comprising (a) heat shock protein and/or alpha-2-macroglobulin, and (b) antigenic proteins wherein said population of complexes were produced by a method comprising (i) exposing a protein preparation comprising at least 50% of the different proteins or at least 50 different proteins

present in antigenic cells to ATP, guanidium hydrochloride, and/or acidic conditions, to produce a population of antigenic peptides; (ii) recovering the population of antigenic peptides; and (iii) complexing the population of antigenic peptides to heat shock protein or alpha-2-macroglobulin; and

a second container containing a non-heat shock protein and non-alpha-2-macroglobulin-based treatment modality.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/06807

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A01N 37/18; A61K 38/02; A23J 1/00
US CL : 424/193.1, 194.1, 195.11, 196.11, 197.11, 277.1, 278.1; 530/412, 413, 828; 514/2, 21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/193.1, 194.1, 195.11, 196.11, 197.11, 277.1, 278.1; 530/412, 413, 828; 514/2, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 6,168,793 B1 (SRIVASTAVA) 2 January 2001 (2.01.2001) column 8, lines 45-49, column 10 lines 31-34 and 61-67. | 1-6,8,10,15-25, 27,29,32-39 |
| Y | US 6,162,436 A (SRIVASTAVA) 19 December 2000 (19.12.2000), column 34, lines 47-62. | 1-6,8,10,15-25, 27,29,32-39 |
| A | US 6,136,315 A (SRIVASTAVA) 24 October 2000 (24.10.2000), see entire document. | 1-39 |
| A | BINDER, R.J et al. Adjuvanticity of alpha 2-Macroglobulin, an Independent Ligand for the Heat Shock Protein Receptor CD91. J. Immunol. April 2001, Vol. 166, No. 8, pages 4968-4972. | 1-6, 11-39 |
| A | BINDER, R.J et al. Naturally Formed or Artificially Rreconstituted Non-Covalent Alpha2-Macroglobulin-Peptide Complexes Elicit CD91-Dependent Cellular Immunity. Cancer Immun. December 2002, Vol. 2, pages 16-24, see entire document. | 1-6, 11-39 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 June 2003 (25.06.2003) | 17 JUL 2003 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US<br>Commissioner for Patents<br>P.O. Box 1450<br>Alexandria, Virginia 22313-1450<br>Facsimile No. (703)305-3230 | Authorized officer<br>Christopher Yaen<br>Telephone No. 703-308-0196 |

Form PCT/ISA/210 (second sheet) (July 1998)

**INTERNATIONAL SEARCH REPORT**

PCT/US03/06807

**Continuation of B. FIELDS SEARCHED Item 3:**
STN DATABASE, MEDLINE, CANCERLIT, BIOSIS, CONFSCI, CAPLUS, EMBASE, USPATFULL, PCTFULL, SCISEARCH, WEST